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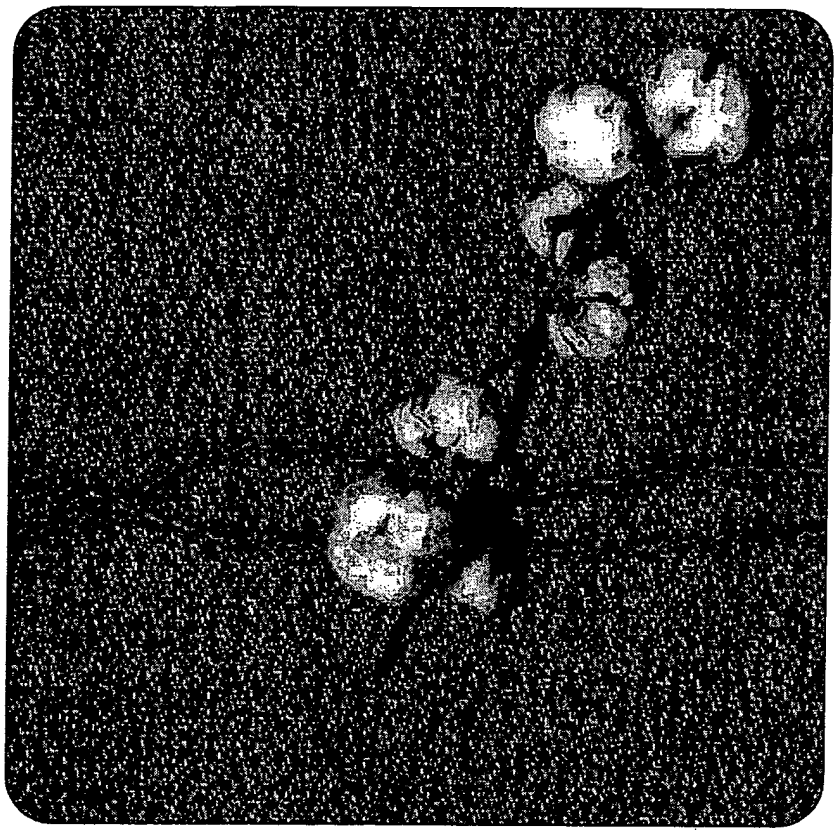

Arja Miettinen-Oinonen

Trichoderma reesei strains for production of cellulases for the textile industry

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Arja Miettinen-Oinonen

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Abstract

Trichoderma reesei is a biotechnically important filamentous fungus used commercially in enzyme production. *T. reesei* is also one of the best known cellulolytic organisms, producing readily and in large quantities a complete set of extracellular cellulases for the degradation of crystalline cellulose. In addition to *T. reesei*, a wide variety of other bacteria and fungi also produce cellulolytic enzymes. Cellulases originating from various organisms and having different characteristics are used industrially in many applications, such as in the textile industry in finishing of denim fabric to impart a stonewashed appearance (biostoning) and in biofinishing of cotton.

In this work *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles were constructed for specific industrial applications, *i.e.* biostoning and biofinishing of cotton. The production of *T. reesei* endoglucanase II (EGII), cellobiohydrolases I and II (CBHI and CBHII) was improved in separate strains. Strains producing high amounts of EGI and EGII without CBHs or CBHI and CBHII without the main EGs were also constructed. The cellulase genes were expressed under the powerful *T. reesei cbh1* promoter; in a transformant overproducing both CBHI and CBHII, the *cbh2* promoter was also used for *cbh2* expression. The level of endoglucanase activity produced by the EGII-overproducing transformants correlated with the copy number of the *egl2* expression cassette. Production of the major secreted cellulase CBHI was increased up to 1.5-fold and production of CBHII fourfold compared with the parent strain. In transformants overproducing both CBHI and CBHII, production of CBHI was increased up to 1.6-fold and production of CBHII up to 3.4-fold as compared with the host strain and approximately similar amounts of CBHII protein were produced by using the *cbh1* or *cbh2* promoters.

The enzyme preparation with elevated EGII content most clearly improved the biostoning of denim fabric and the biofinishing of cotton fabric. Better depilling and visual appearance were achieved with the enzyme preparation having an elevated CBHII content compared to the wild type preparation in biofinishing of cotton, but the improvement was not as pronounced as in the case of the EGII preparation.

Novel neutral cellulases were demonstrated to have potential in biostoning. The cellulase preparation of the thermophilic fungus *Melanocarpus albomyces* was found to be effective in releasing dye from indigo-dyed denim and to cause low backstaining at neutral pH. *M. albomyces* produces at least three cellulases and these cellulases with an effect on biostoning were purified and the genes encoding them were cloned and sequenced. *Ma* 20 kDa EGV (*Ma* Cel45A) belongs to the glycosyl hydrolase family 45 and the 50 kDa EGI (*Ma* Cel7A) and CBHI (*Ma* Cel7B) to family 7. None of the cellulases harbours a cellulose binding domain. Especially purified *Ma* Cel45A performed well in biostoning. The *Ma* cellulases were produced in *T. reesei* under the *T. reesei cbh1* promoter for biostoning applications. The endoglucanase production levels of *Ma cel45A*- and *cel7A*-transformants were several times higher than those of the parental *M. albomyces* strain. The cellulase preparation produced by the recombinant *Ma cel45A* transformant performed well at neutral pH in the finishing of denim fabric and caused considerably less backstaining than the acid cellulase product of *T. reesei.*

Preface

This work was carried out at Primalco Ltd. Biotec and Röhm Enzyme Finland Oy. I wish to thank Kari Edelmann and Ralf Lundell for providing excellent working facilities. I thank Professor Hannu Saarilahti from the Division of Genetics in Helsinki University for his support during the final stages of my studies. I am grateful to Professor Helena Nevalainen and Professor Georg Gübitz for reviewing this thesis work and for useful comments.

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List of publications

This work is based on the following articles, referred to in the text by the Roman numerals given below. Additional data published in US Patent 5,874,293 (http://www.uspto.gov, Miettinen-Oinonen, A., Elovainio, M. and Suominen, P.: Cellulase composition for treating cellulose-containing textile material, filed January 1997) is also presented.

I. Miettinen-Oinonen, A. and Suominen, P. 2002. Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. Applied and Environmetal Microbiology 68:3956–3964.

II. Miettinen-Oinonen, A., Paloheimo, M., Lantto, R. and Suominen, P. 2004. Enhanced production of cellobiohydrolases in *Trichoderma reesei* and biofinishing of cotton fabric with the new preparations. Submitted for publication in Journal of Biotechnology.

III. Miettinen-Oinonen, A., Londesborough, J., Joutsjoki, V., Lantto, R. and Vehmaanperä, J. 2004. Three cellulases from *Melanocarpus albomyces* with applications in the textile industry. Enzyme and Microbial Technology 34:332–341.

IV. Haakana, H., Miettinen-Oinonen, A., Joutsjoki, V., Mäntylä, A., Suominen, P. and Vehmaanperä, J. 2004. Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei.* Enzyme and Microbial Technology 34:159–167.

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Abbreviations

ACE	Activator of cellulase expression
BGL	β-glucosidase
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
cbh	Gene encoding cellobiohydrolase
Cel	Cellulase
cel	Gene encoding cellulase
CD	Catalytic domain
CMC	Carboxymethyl cellulose
CRE	Carbon catabolite repressor element
DP	Degree of polymerisation
3D-structure	Three-dimensional structure
ECU	Activity against hydroxyethyl cellulose
EG	Endoglucanase
egl	Gene encoding endoglucanase
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FPU	Filter paper-hydrolyzing activity
GH	Glycoside hydrolase
HEC	Hydroxyethyl cellulose
ORF	Open reading frame
PCR	Polymerase chain reaction
pdi	Gene encoding protein disulphide isomerase
pgk	Gene encoding phoshoglycerate kinase
pki	Gene encoding pyruvate kinase
UPR	Unfolded protein response

1. Introduction

The genus *Trichoderma* comprises a group of filamentous ascomycetes that are widely used in industrial applications because of their ability to produce extracellular lignocellulose-degrading hydrolases in large amounts. Enzymes secreted by *Trichoderma* have received widespread industrial interest, leading to commercial applications in the textile industry (Galante *et al.*, 1998a, Cavaco-Paulo and Gübitz, 2003, Nierstrasz and Warmoeskerken, 2003), the food and feed industries (Galante *et al.*, 1998b) and the pulp and paper industry (Buchert *et al.*, 1998). *Trichoderma reesei* has the capacity to secrete enzymes in high yields and this property can be exploited when using *T. reesei* as an industrial host for homologous and heterologous enzyme production (Mäntylä *et al.*, 1998, Penttilä, 1998, Penttilä *et al.*, 2004). However, there is a continuous need to improve enzyme production processes in order to enhance protein yields and improve the production economics.

Cellulolytic enzymes hydrolyze cellulose and are produced by a wide variety of bacteria and fungi, *T. reesei* being one of the best-known cellulolytic organisms. Cellulases are industrially important enzymes (reviewed by Schülein, 2000) with a current market value of about 190 million US $ (Nierstrasz and Warmoeskerken, 2003). In the textile industry cellulase enzymes are tools for the fabric and garment finisher to produce higher value products, as cellulases clean fuzz and prevent formation of pills on the surface of cotton garments. Cellulases can also be used in denim finishing to create a fashionable stonewashed appearance in denim cloths in a process called biostoning. About 10 % of textile finishing of cellulose materials is estimated to be performed by cellulases and approximately 80 % of the 1.8 mrd pairs of denim jeans produced annually are finished with cellulases as an alternative to pumice stones (Buchert and Heikinheimo, 1998). Cellulases from the fungi *T. reesei* and *Humicola insolens* are widely used in the textile industry and depending on the desired effect different types of cellulases are utilized. In the detergent industry cellulases are used to clean cotton garments or to brighten faded coloured garments by removing fuzz (Maurer, 1997). In animal feed cellulases are utilized together with other hydrolases in the degradation of non-starch polysaccharides to improve feed conversion rates (Galante *et al.*, 1998b). The food industry uses cellulases together with other plant cell wall-degrading enzymes in fruit and

vegetable processing (Urlaub, 2002). Enzymatic hydrolysis of biomass to sugars for subsequent ethanol production has also been a major research area during recent years (Himmel *et al.*, 1999). Cellulases have potential in the pulp and paper industry, e.g. in deinking to release ink from fibre surfaces and in improving pulp drainage (Suurnäkki *et al.*, 2004). Within the forest industry cellulases have been shown to be effective in decreasing the energy consumption of mechanical pulping (Pere *et al.*, 2002). Cellulases have been found to increase the alkali solubility of treated pulp and directly alkali soluble cellulose has been obtained with specific cellulase compositions (Vehviläinen *et al.*, 1996, Rahkamo *et al.*, 1996). This property can be utilized in developing new, environmentally benign processes for manufacturing cellulosic articles such as films and fibres. The wide spectrum of industrial uses for cellulases establishes a need for commercial cellulase products containing different cellulase components and functioning optimally in different pH and temperature ranges.

1.1 Enzymatic degradation of cellulose

1.1.1 Cellulose

Cellulose is the main constituent of plants and thus the most abundant biopolymer on earth. Native cellulose is an unbranched homopolysaccharide consisting of D-glucose residues linked by β-1,4-glycosidic bonds to form a linear polymeric chain (Figure 1). The smallest repetitive unit in cellulose is cellobiose, which consists of two glucose units. In nature, cellulose chains have a degree of polymerization (DP) of approximately 10 000 glucose units in wood cellulose and 15 000 in native cotton cellulose (Sjöström, 1981). In crystalline cellulose the chains adhere to each other by hydrogen bonding and van der Waals forces to form highly insoluble structures. In addition to crystalline regions, native cellulose contains less-ordered amorphous or paracrystalline regions (Teeri, 1997 and refs there). Six polymorphs of cellulose (I, II, III_I, III_{II}, IV_I and IV_{II}) have been documented (reviewed in O'Sullivan, 1997). Cellulose I, or native cellulose, the form found in nature, exists further in two crystalline forms termed celluloses Iα and Iβ. Cellulose II can be obtained from cellulose I by regeneration or merceration. Celluloses III_I and III_{II} are formed from celluloses I and II by treatment with liquid ammonia. Polymorphs IV_I and IV_{II} may be prepared by heating celluloses III_I and III_{II}.

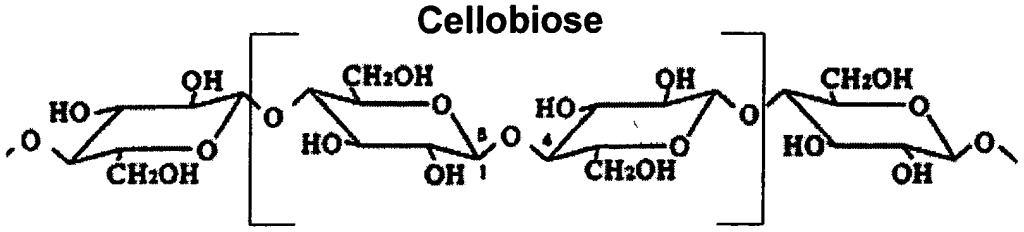


Figure 1. The cellulose chain.

1.1.1.1 Cotton

Cotton fibre originates from the seed hair of plants of the genus *Gossypium*. The fibre appears as a long, irregular, twisted, and flattened tube (Morton and Hearle, 1997). In cross-section, mature fibres have the form shown in Figure 2. The outermost layer is the cuticle, a thin film of fats, pectin and waxes (Trotman, 1993). Beneath this is the primary wall, composed mainly of cellulose in which the fibrils are arranged in a criss-cross pattern. The bulk of the fibre is made up of the secondary wall, composed of cellulose. The secondary wall is differentiated into three discernible zones. In the centre is a narrow collapsed lumen.

In addition to cellulose, raw cotton fibre contains impurities such as oil, waxes, pectins, proteins and simpler related nitrogenous compounds, organic acids, mineral matter, and natural colouring agents (Trotman, 1993). 85–90 % of raw cotton is composed of cellulose.

According to Rouette (2002), one cotton fibre consists of 15 000 microfibrils, and one microfibril contains 400 elementary fibrils. In one elementary fibril 100 cellulose chains are arranged in 6–8 packages. Cotton fibres consist of crystalline fibrils between which amorphous unordered regions are found. Approximately 70 % of the cotton fibre is crystalline (Needles, 1986, Morton and Hearle, 1997). Voids, spaces, and structural irregularities occur in the amorphous areas, whereas the cellulose chains in the crystalline regions are

tightly packed. Penetration of dyestuffs and chemicals occurs more readily in the amorphous regions (Needles, 1986).

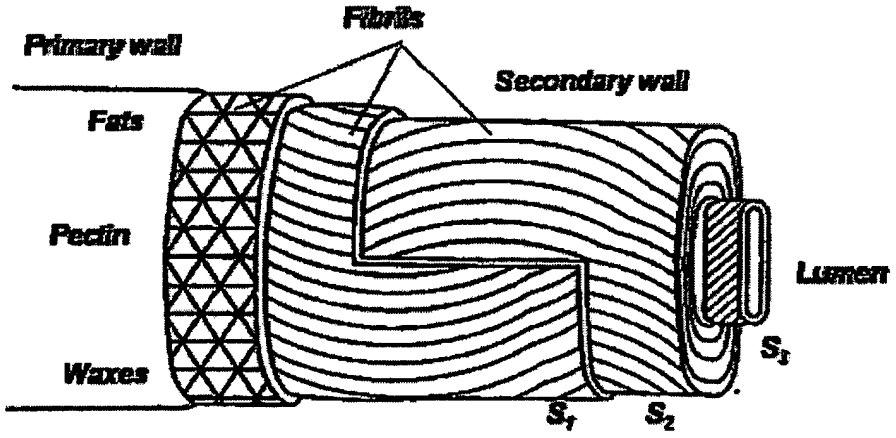


Figure 2. Morphological structure of the cotton fibre, S = secondary wall, S_1–S_3 represent different layers of secondary wall (Morton and Hearle, 1997).

1.1.2 Hydrolysis of cellulose

A wide variety of bacteria and fungi produce cellulolytic enzymes able to hydrolyze cellulose. However, relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (reviewed in Bhat and Bhat, 1997). Saprophytic filamentous fungi produce three categories of cellulases, endoglucanases (EG, 1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4), cellobiohydrolases (also called exoglucanases, CBH, 1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91), and β-glucosidases (cellobiase or β-D-glucoside glucohydrolase, BGL; EC 3.2.1.21) in order to degrade insoluble cellulose into glucose. Endoglucanases cleave bonds along the length of the cellulose chains in the middle of the amorphous regions, resulting in a decrease in the DP of the substrate (reviewed in Teeri and Koivula, 1995, Teeri, 1997) (Figure 3). Cellobiohydrolases are processive enzymes, initiating their action from the ends of the cellulose chains. They attack the crystalline parts of the substrate, produce primarily cellobiose, and decrease the DP of the substrate only very slowly. The hydrolysis of the glycosidic bonds occurs by

general acid catalysis with the involvement of two carboxylic amino acids (reviewed in Koivula *et al.*, 1998). The cellobiohydrolases act synergistically with each other and with endoglucanases: *i.e.* mixtures have a higher activity than the sum of the activities of the individual enzymes acting alone. Cellulolytic fungi generally produce two different CBHs. *Trichoderma reesei* CBHI and CBHII have opposite chain-end specificities with regard to liberation of cellobiose from the end of the cellulose glucan chains. *T. reesei* CBHI attacks the reducing end, whereas CBHII acts at the non-reducing end (Barr *et al.*, 1996). Cellobiohydrolase and endoglucanases act together to hydrolyze cellulose to small cello-oligosaccharides. In the final cellulose hydrolysis step β-glucosidases hydrolyze the soluble oligosaccharides and cellobiose to glucose.

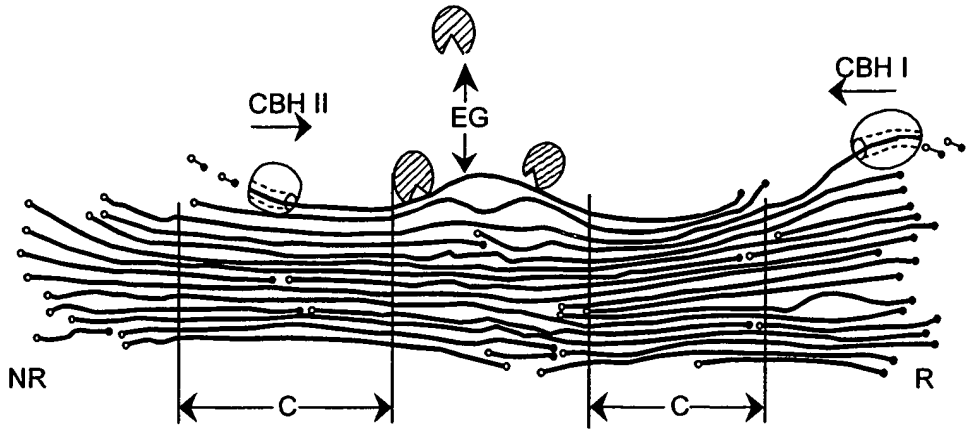


Figure 3. Mechanism of enzymatic hydrolysis of cellulose (Teeri, 1997). The two cellobiohydrolases (CBH) attack the crystalline areas at the opposite chain ends and endoglucanases (EG) in the middle of the more disordered regions of cellulose. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the non-reducing ends. C indicates the highly ordered crystalline regions.

Degradation of native cotton cellulose by *T. reesei* EGI and CBHII has been studied by analyzing the insoluble cellulose fragments remaining after enzymatic hydrolysis (Kleman-Leyer *et al.*, 1996). During the incubation EGI alone solubilized the cellulose (increase of weight loss, decrease of DP), but CBHII did not depolymerize the cellulose. A synergistic effect was observed in reducing sugar production. It was concluded that EGI degrades cotton cellulose

by selectively cleaving through the microfibrils at the amorphous sites, whereas CBHII releases soluble sugars from the EGI-degraded cotton cellulose. After cellulase treatment without additional mechanical action, purified EGI, EGII, CBHI and CBHII from *T. reesei* all reduced the molecular weight of powder formed from cotton poplin (Heikinheimo *et al.*, 2003). When mechanical action was combined with the enzyme treatments, only EGII reduced the molecular weight. Rousselle *et al.* (2002) observed changes in molecular weight distribution, weight and strength losses, fibre pore distribution and hydrogen bonding patterns of cotton cellulose after treatment of the cotton fabric with total cellulase of *T. reesei.*

1.2 Fungal cellulolytic enzymes

The cellulase systems of the aerobic fungi *Trichoderma reesei, Trichoderma viride, Penicillium pinophilum, Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani, Talaromyces emersonii, Trichoderma koningii* and *Rhizopus oryzae* are well characterized (reviewed in Bhat and Bhat, 1997, Murashima *et al.*, 2002). Some thermophilic aerobic fungi (see 1.2.3.) and mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis*) (Bhat and Bhat, 1997) also produce cellulases.

1.2.1 Classification of cellulases

Cellulases belong to the O-glycoside hydrolases (EC 3.2.1.–), which are a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUB Enzyme Nomenclature (EC-number) is based on the type of reaction that enzymes catalyze and on their substrate specificity. According to the new classification glycoside hydrolases (GHs) are classified in families based on amino acid sequence similarities (Henrissat, 1991, Henrissat and Bairoch, 1993, Henrissat and Bairoch, 1996, Bourne and Henrissat, 2001). There is a direct relationship between sequence and folding (Henrissat, 1991). There are two major cleavage mechanisms for glycoside hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point, and the mechanism appears to be conserved within each family (reviewed in Henrissat and Bairoch, 1996). Thus the catalytic domains of GHs in one family have the

same three-dimensional fold and exhibit the same stereospecificity of hydrolysis; for example retaining in family 5 and inverting in family 6 (Henrissat *et al.*, 1998). There are currently (as in July 2004) 97 families of GHs (URL http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), with cellulases being found in at least 13 of them. Some 450 cellulase genes (derived from all kinds of microorganisms) exist in public domain databases (Schülein, 2000).

1.2.2 Modular structures of cellulases

Many of the fungal hydrolases are modular proteins, and all of them contain a catalytic domain (CD). In addition to the CD, GHs may contain a carbohydrate-binding module (CBM), which is defined as contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity (Bourne and Henrissat, 2001). CBMs were previously classified as cellulose-binding domains (CBD), based on the initial discovery of domains that bind to cellulose (Tomme *et al.*, 1995a, b). The CBMs also form sequence-based families and are numbered with arabic numerals (Bourne and Henrissat, 2001). Currently (as in July 2004) there are 39 families of CBM (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), with CBMs of cellulases being found in at least 13 of them.

CBMs in most fungal cellulases are located at either terminus of the catalytic domain and are connected to CD by an often heavily glucosylated linker. The characterized role of the CBM is to mediate binding of the enzyme to the insoluble substrate. The overall binding efficiency of the cellulase is much enhanced by the presence of the CBM and the enhanced binding clearly correlates with better hydrolytic activity towards insoluble cellulose (reviewed in Linder and Teeri, 1997). Removal of the CBM has little influence on activity of cellulases towards soluble subtrates, although their binding properties and catalytic activity towards insoluble cellulose is clearly decreased (reviewed in Linder and Teeri, 1997). For example the presence of CBM in *T. reesei* cellulases is reported to enhance the enzymatic hydrolysis of insoluble isolated cellulose and chemical pulp (Suurnäkki *et al.*, 2000). CBMs allow cellulases to act on crystalline cellulose by destabilising the hydrogen bond structure of cellulose, making the polysaccharide chains more accessible to the catalytic domain (Quentin *et al.*, 2003). Fungal CBMs are also reported to contribute to the non-hydrolytic disruption of cotton fibre.

1.2.3 Thermophilic cellulases

Thermophilic fungi have a growth temperature minimum at or above 20°C and a growth temperature maximum at or above 50°C. Cellulases have been isolated from various thermophilic fungi, e.g. *Chaetomium thermophile, Humicola insolens, Humicola grisea* var. *thermoidea, Myceliophthora thermophila, Talaromyces emersonii* and *Thermoascus aurantiacus* (reviewed in Maheshwari *et al.*, 2000). The identified endoglucanases (30 to 100 kDa) of thermophilic fungi are thermostable, with optimal activity between 55 and 80°C at pH 5.0 to 5.5 and with carbohydrate contents from 2 to 50 % (reviewed in Maheshwari *et al.*, 2000) (Table 1). The exoglucanases (40 to 70 kDa) are reported to have an activity optimum between 50 and 75°C and they are thermostable.

Melanocarpus albomyces is a thermophilic ascomycete with alternative names *Myriococcum albomyces* and *Thielavia albomyces* (Maheshwari *et al.*, 2000). Qureshi *et al.* (1980) reported M*elanocarpus albomyces* to be a cellulolytic fungus, but according to Maheshwari and Kamalam (1985) it is unable to utilize cellulose. According to El-Gindy (1991) and Chung (1971) *Myriococcum albomyces* produces cellulases. *Melanocarpus albomyces* has been reported to produce xylanase (Prabhu and Maheshwari, 1999) and laccase (Kiiskinen *et al.*, 2002) activities.

Table 1. Properties of various endoglucanases of thermophilic fungi (reviewed in Maheshwari et al., *2000).*

Fungus	Optimal pH	Optimal temp. (°C)	Mol mass kDa	Carbo-hydrate %
*Chaetomium thermophile**	5–6	55–60	36–41	n.r.
Humicola grisea var. thermoidea	5	n.r.	63	n.r.
Humicola insolens	5–5.6	50	45–57	16–39
*Myceliophthora thermophila***	4.8	65	100	n.r.
Talaromyces emersonii	5.5–5.8	75–80	35	27.7–50.8
Thermoascus aurantiacus	2.9–4.5	65–76	32–34	1.7–1.8

* Other names *C. thermophilum, C.thermophilium*

** Other names *Sporotrichum thermophilum / thermophile, Chrysosporium thermophilum*

n.r.= Not reported

1.2.4 Structure of fungal cellulases used in cotton finishing

Fungal cellulases from various sources are currently used in denim finishing to impart a stone-washed appearance and in biofinishing (more details in 1.5. and Table 6).

Trichoderma reesei secretes a complete mixture of cellulolytic enzymes for degradation of crystalline cellulose to glucose. It is an asexually reproducing filamentous fungus, isolated from cotton canvas in the Solomon Islands during World War II (Kuhls *et al.*, 1996). *T. reesei* is an asexual clonal line derived from a population of the tropical saprophytic Ascomycete *Hypocrea jecorina* (Kuhls *et al.*, 1996). The cellulolytic system of *T. reesei* is composed of at least eight endoglucanases and two cellobiohydrolases (Table 2). Seven β-glucosidases, which hydrolyze cellobiose to glucose, have been identified (Table 2). *T. reesei* cellulases generally have a modular structure consisting of a cellulose binding module (CBM, previously called CBD) at either end of the polypeptide chain, connected to the catalytic domain by a linker region (Shoemaker *et al.*, 1983, Teeri *et al.*, 1987, Penttilä *et al.*, 1986, Saloheimo *et al.*, 1988, 1994, 1997). *T. reesei* EGIII lacks the CBM and the linker region (Ward *et al.*, 1993, Okada *et al.*, 1998). The most recently identified genes encoding endoglucanases are *cel74a, cel61b* and *cel5b* (Foreman *et al.*, 2003). Cel74a is the first member of GH family 74 to be found in *T. reesei*, and it includes a C-terminal carbohydrate-binding domain of the CBM1 family. Cel61B has no CBM. Cel5B may represent a membrane-bound endoglucanase. The 3D-structures of the catalytic cores of CBHI (Cel7A, Divne *et al.*, 1994), CBHII (Cel6A, Rouvinen *et al.*, 1990), EGI (Cel7B, Kleywegt *et al.*, 1997) and EGIII (Cel12A, Sandgren *et al.*, 2001) and of the cellulose binding domains of CBHI (Cel7A, Kraulis *et al.*, 1989) and EGI (Cel7B, Mattinen *et al.*, 1998) have been solved. The active site of the cellobiohydrolases CBHI and CBHII is located in a tunnel, whereas the endoglucanases EGI and EGIII have the active site in an open cleft. An additional protein, swollenin (encoded by a gene *swo1*), which is assumed to disrupt e.g. the structure of cotton fibres, has been described (Saloheimo *et al.*, 2002b). This protein has an N-terminal fungal type CBM connected by a linker to the expansin-like domain.

The saprophytic thermophilic fungus *Humicola insolens* produces at least six different endoglucanases and two cellobiohydrolases, genes for which have all

been cloned (Dalboge and Heldt-Hansen, 1994, Schülein, 1997, Schülein *et al.*, 1998, Murashima *et al.*, 2000) (Table 3). At least five cellulase genes have been cloned from *Fusarium oxysporium* (Sheppard *et al.*, 1994) (Table 4). Cellulase genes have been cloned from the fungal ascomycetes *Myceliophthora thermophila, Acremonium* sp., *Macrophomina phaseolina* and *Thielavia terrestris* and from the fungal basidiomycete *Crinipellis scabela* (Schülein *et al.*, 1996, 1998) (Table 4). Two family 45 endoglucanase genes were found in *Acremonium* sp. (Schülein *et al.*, 1998).

3D-structures of *Humicola* EGI (Cel7B, Mackenzie *et al.*, 1998) and EGV core (Cel45, Davies *et al.*, 1995) have been published. The 3D-structure of *Fusarium* EGI has been solved (Sulzenbacher *et al.*, 1996). The crystal structure of the family 45 cellulase (*Ma* 20kDa EG) from *Melanocarpus albomyces* has recently been determined (Hirvonen and Papageorgiou, 2002, Valjakka and Rouvinen, 2003).

Table 2. Cellulolytic system of T. reesei.

Enzyme	Gene	GenBank™ accession no.	Length[1]	Molecular mass, kDa[1]	Structural organisation[3]	Ref.
CEL7A (CBHI)	*cbh1/cel7a*	P00725[12]	513	59-68		Shoemaker *et al.*, 1983, Teeri *et al.*, 1983
CEL6A (CBHII)	*cbh2/cel6a*	M16190	471	50-58		Teeri *et al.*, 1987
CEL7B (EGI)	*egl1/cel7b*	M15665	459	50-55		Penttilä *et al.*, 1986
CEL5A (EGII)	*egl2/cel5a*	M19373	418	48		Saloheimo *et al.*, 1988
CEL12A (EGIII)	*egl3/cel12a*	AB003694	234	25		Ward *et al.*, 1993, Okada *et al.*, 1998
CEL61A (EGIV)	*egl4/cel61a*	Y11113	344	34[2]		Saloheimo *et al.*, 1997
CEL45A (EGV)	*egl5/cel45a*	Z33381	242	23[2]		Saloheimo *et al.*, 1994
EGVI[4]				95-105		Bower *et al.*, 1998a
CEL74A (EG)[5,8]	*cel74a*	AY281371	838	87[2]	Contains CBM[10]	Foreman *et al.*, 2003
CEL61B (EG)[5,8]	*cel61b*	AY281372	249	27[2]	No CBM[10]	Foreman *et al.*, 2003
CEL5B (EG)[5,9]	*cel5b*	AY281373	438	47		Foreman *et al.*, 2003
CEL3A (BGLI)	*bgl1/cel3a*	U09580	744	75		Barnett *et al.*, 1991, Mach, 1993
CEL1A (BGLII)	*bgl2/cel1a*	AB003110	466	52		Takashima *et al.*, 1999, Saloheimo *et al.*, 2002a
CEL3B (BGL)[5,6]	*cel3b*	AY281374	874	94[2]		Foreman *et al.*, 2003
CEL3C (BGL)[5,7]	*cel3c*	AY281375	833	91[2]		Foreman *et al.*, 2003
CEL1B (BGL)[5,7]	*cel1b*	AY281377	484	55[2]		Foreman *et al.*, 2003
CEL3D (BGL)[5,7]	*cel3d*	AY281378	700	77[2]		Foreman *et al.*, 2003
CEL3E (BGL)[5,6]	*cel3e*	AY281379	765	83[2]		Foreman *et al.*, 2003

[1]Length of the protein including the signal peptide, amino acid residues, [2] Molecular mass calculated from the amino acid sequence, [3] = CD, β = linker, ■ = CBM [4] Described on the protein level,

Table 3. Cellulolytic system of H. insolens.

Enzyme	Amino acid residues			Reference
	Core	Linker	CBM	
CBHI (Cel7)	437	42	36	Dalboge and Heldt-Hansen, 1994
CBHII (Cel6)	366	45	38	Schülein, 1997
EGI (Cel7)	398	15		Schülein, 1997
EGII (Cel5)	303	33	36	Dalboge and Heldt-Hansen, 1994
EGIII (Cel12)	224			Dalboge and Heldt-Hansen, 1994
EGV (Cel45)	213	33	38	Rasmussen *et al.*, 1991
EGVI (Cel6)	346	37	37	Dalboge and Heldt-Hansen, 1994
EG (NCE4)	Total 284			Murashima *et al.*, 2000

Table 4. Cellulases of Acremonium sp., C. scabela, M. thermophila, M. phaseolina, T. terrestris and F. oxysporium.

Organism	Enzyme	Gene	Amino acid residues	Reference
Acremonium sp.	EGV (Cel45)	n.r.	208 core, 36 linker, 35 CBM	Schülein *et al.*, 1998*
	EG	n.r.	295	Schülein *et al.*, 1996*
	EG	n.r.	349	Schülein *et al.*, 1996*
	CBHI (partial)	n.r.	160	Lange *et al.*, 2003*
	CBHI (partial)	n.r.	164	Lange *et al.*, 2003*
C. scabela	EGV (Cel45)	n.r.	204, no linker or CBM	Schülein *et al.*, 1998*
	EG	n.r.	226, no linker or CBM	Schülein *et al.*, 1996*
M. thermophila	EGI (Cel7)	n.r.	436	Schülein *et al.*, 1998*, Osten and Schülein, 1999
	EGV (Cel45)	n.r.	207	Schülein *et al.*, 1998*
	EG	n.r.	225, no linker or CBM	Schülein *et al.*, 1996*
M. phaseolina	EGV (Cel45)	n.r.	203, no linker or CBM	Schülein *et al.*, 1998*
	EGI (Cel5)	*egl1*	333	Wang and Jones, 1995b
	EGII (Cel5)	*egl2*	368, no linker or CBM	Wang and Jones, 1995a
T. terrestris	EGV (Cel45)	n.r.	211 core, 30 linker, 37 CBM	Schülein *et al.*, 1998*
	EG	n.r.	299	Schülein *et al.*, 1996*
F. oxysporium	EGI (Cel7B)	*Cfam1*	427	Sheppard *et al.*, 1994
	EGV (Cel45)	*Kfam1*	312 core, 34 linker, 31 CBM	Sheppard *et al.*, 1994
	n.r.	*Ffam1*	323 core, 32 linker, 31 CBM	Sheppard *et al.*, 1994
	CBHI (Cel7A)	*Cfam2*	441 core, 43 linker, 31 CBM	Sheppard *et al.*, 1994
	EG B	*Bfam1*	392 core, 39 linker, 31 CBM	Sheppard *et al.*, 1994

n.r. = not reported, * Limited information is given in the references.

1.3 Production of cellulases by *T. reesei*

Filamentous fungi are used for the commercial production of various metabolites. The main genera of filamentous fungi used in industry are *Trichoderma* (enzymes), *Aspergillus* (enzymes, organic acids, fermented foods), *Mucor* (enzymes), *Rhizopus* (enzymes), *Penicillium* (antibiotics, cheese) and *Cephalosporium* (antibiotics) (Nevalainen, 2001).

T. reesei has several advantages for industrial-scale production of homologous and heterologous cellulases and proteins in general. *T. reesei* is easy and inexpensive to cultivate and it is currently grown in fermenters up to 230 m^3, which shows that its fermenter technical properties are good and that it is not susceptible to contamination (Penttilä, 1998). *T. reesei* has a secretory machinery with protein modifications typical of eukaryotes (Palamarczyk *et al.*, 1998). *T. reesei* is considered to be a safe production organism, because it is non-pathogenic to healthy humans and does not produce mycotoxins or antibiotics under the conditions used for enzyme production (Nevalainen *et al.*, 1994, Nevalainen and Neethling, 1998). *T. reesei* strains producing cellulases have been evaluated as belonging to Group I (low-risk level) microorganisms (EC Directive 90/219/EEC) (Penttilä *et al.*, 2004). *T. reesei* produces extracellular proteins and cellulases naturally in large quantities. Industrial *T. reesei* strains have been obtained by using classical mutagenesis techniques to enhance the release of extracellular proteins in general, to increase cellulase production levels (reviewed in Mäntylä *et al.*, 1998, Durand *et al.*, 1988a) and to obtain protease-deficient mutants (Mäntylä *et al.*, 1994). *T. reesei* mutant strains have been reported to produce up to 40 g/l proteins in the culture medium in optimized cultivation conditions (Durand *et al.*, 1988b). Of the extracellular proteins produced by *T. reesei* the main part is composed of cellulases. CBHI represents approximately 50 % of all protein secreted by wild-type *T. reesei* (Penttilä, 1998). The proportion of CBHI is about 60 %, CBHII 25 % and of endoglucanases 15 % of the cellulase proteins of *T. reesei* (Gritzali and Brown, 1979). CBHII accounts for the major portion of the conidial-bound cellulases (Stangl *et al.*, 1993). Production of EGI is reported to represent 5 to 10 % of the secreted protein (Penttilä *et al.*, 1987), but EGII is proposed to account for most of the endoglucanase activity produced by *T. reesei* (Suominen *et al.*, 1993).

Successful mutagenesis, screening and process development has led to *T. reesei* strains feasible for commercial cellulase production, but genetically modified strains have further been developed from the classical strains for various applications (Mäntylä *et al.*, 1998). The essential tools needed for construction of industrial production strains by genetic engineering have been developed for *Trichoderma.* These include different mutant strains that are used as hosts for recombinant protein production, transformation techniques with a variety of selection markers (reviewed in Mach and Zeilinger, 1998, Hazell *et al.*, 2000), methods for gene targeting and replacement (Suominen *et al.*, 1993) and a series of expression cassettes and transcription termination sequences (reviewed in Nevalainen, 2001). Recent developments in sequencing of the genome of *T. reesei* (completed, not published, http://www.jgi.doe.gov) and EST sequencing of *T. reesei* (http://trichoderma.iq.usp.br/TrEST.html), as well as the first transciptional profiling experiments with available EST sequences (Chambergo *et al.*, 2002) and the first proteomics methods (Lim *et al.*, 2001), will provide means to further improve the efficient protein production system of *Trichoderma* (Penttilä *et al.*, 2004).

1.3.1 Regulation of cellulase gene expression

In *T. reesei* the production of cellulases is controlled at the transcriptional level depending on the available carbon source (Kubicek and Penttilä, 1998). Transcription of the major components of cellulase (CBHI, CBHII, EGI, EGII, EGIII, EGIV, EGV, Cel61B, Cel74A) is induced by cellulose and a variety of disaccharides including lactose, β-cellobiono-1,5-lactone, cellobiose, and sophorose (two β-1,2-linked glucose units) (Kubicek *et al.*, 1993, Saloheimo *et al.*, 1997, Ilmén *et al.*, 1997, Nogawa *et al.*, 2001, Foreman *et al.*, 2003). The best inducing compound known to date is sophorose (reviewed in Mach and Zeilinger, 2003). When glucose, fructose, or glycerol are used as the carbon source, no significant levels of cellulases are produced (Ilmén *et al.*, 1997). In inducing conditions the cellulase genes (*cbh1, cbh2, egl1, egl2, egl4* and *egl5*) have been shown to be expressed coordinately, and expression of the *cbh1* gene has shown to be the highest (Ilmén *et al.*, 1997, Saloheimo *et al.*, 1997). Behaviour of the *egl3* has been studied less, but according to Nogawa *et al.* (2001) it is coordinately transcribed together with the other cellulase genes at least when induced by sophorose. The gene for the putative membrane-bound endoglucanase *cel5b* has different transcriptional regulation as compared to the

other endoglucanases. It is expressed on both glucose and glycerol and is induced only slightly by either cellulose or sophorose (Foreman *et al.*, 2003).

T. reesei cellulase regulation has been analyzed at the molecular level. Cellulase genes are repressed in the presence of glucose by the carbon catabolite repressor CRE1 (Ilmén *et al.*, 1996, Strauss *et al.*, 1995). In addition to CRE1 the transcription factors ACEI and ACEII are known to regulate the cellulase promoters in *T. reesei*. ACEII is known to bind to the *cbh1* promoter (Aro *et al.*, 2001). ACEII is an activator of the main cellulase genes in cellulose-induced cultures (Aro, 2003). ACE1 is also able to bind to the *cbh1* promoter (Saloheimo *et al.*, 2000). Deletion of *ace1* resulted in an increase in the expression of all the main cellulase genes in sophorose- and cellulose-induced cultures, indicating that ACEI acts as a repressor of cellulase expression (Aro *et al.*, 2003). Zeilinger *et al.* (1998) identified a *cbh2* activating element in the 5' regulatory sequences of the *cbh2* gene to which the transcriptional regulator HAP2/3/5 protein complex and an as yet unknown further protein bind and are essential for induction of *cbh2* gene expression (reviewed in Mach and Zeilinger, 2003).

1.3.2 Homologous and heterologous expression

In filamentous fungi the production of heterologous proteins is often limited whereas the production of homologous proteins can be high (Iwashita, 2002). Many fungi can secrete gram per liter amounts of endogenous and heterologous fungal gene products in the culture medium, but attempts to produce high levels of proteins of bacterial, plant and mammalian origin have been less successful (Nevalainen, 2001). The factors limiting the amount of heterologous products synthesized in filamentous fungi have been codon usage, proteolytic processing, protein folding, glycosylation and proteolytic degradation (Nevalainen, 2001). The strategies used for the overproduction of fungal proteins in filamentous fungi have included introduction of multiple copies of the gene of interest, use of the promoters of highly expressed genes, expression at a locus of a highly expressed gene, accomplishing position-independent expression, minimizing the proteolytic degradation and development of improved cultivation media (Verdoes *et al.*, 1995). Additionally especially heterologous gene expression of non-fungal proteins in filamentous fungi has been improved by fusing the

corresponding gene to the 3' end of the homologous gene or a fragment thereof (Gouka *et al.*, 1997, Paloheimo *et al.*, 2003).

T. reesei has excellent capacity to secrete large amounts of proteins. CBHI is the major secreted protein of *T. reesei,* accounting about 50 % of the total secreted proteins (Penttilä, 1998). CBHI is the product of a single gene and thus the *cbh1* promoter is regarded as a strong promoter. According to Ilmén *et al.* (1997) in inducing conditions the steady-state mRNA level of *cbh1* is approximately 1.5 and 3 times more abundant than that of *cbh2* and *egl1* mRNAs, respectively. For these reasons the *cbh1* promoter has been exploited for expressing various homologous hydrolases (reviewed in Mäntylä *et al.*, 1998) and heterologous fungal, bacterial and mammalian proteins (reviewed in Paloheimo *et al.*, 1993, Verdoes *et al.*, 1995, Gouka *et al.*, 1997, Radzio and Kück, 1997, Penttilä, 1998, Bergquist *et al.*, 2002) for various applications. Examples of heterologous products expressed and secreted in *T. reesei* include calf chymosin, antibody Fab fragments and single chain antibodies, interleukin-6, tissue plasminogen activator and heterologous fungal proteins such as laccase, chitinase, acid phosphatase and thermophilic enzymes (reviewed in Penttilä *et al.*, 2004). Production of proteins from unrelated species has been shown to benefit from production as fusions to a well-expressed native protein such as CBHI (Penttilä *et al.*, 2004). Successful production of a bacterial xylanase in *T. reesei* has been demonstrated by altering the codon usage pattern (Te'o *et al.*, 2000).

In *T. reesei,* secreted amounts of heterologous fungal enzymes have reached grams per litre level (Paloheimo *et al.*, 1993), although the levels of some proteins such as lignin peroxidases and certain laccases have remained low (Penttilä *et al.*, 2004). For example *Hormoconis resinae* glucoamylase was expressed at a rather high level (0.7 g/l) in *T. reesei* under the *cbh1* promoter (Joutsjoki *et al.*, 1993). Expression of proteins as a fusion to native protein has also been used in production of heterologous fungal proteins in *T. reesei.* As an example expression of *xyn2,* encoding a family 11 xylanase of the thermophilic fungus *Humicola grisea* var. *thermoidea,* as a fusion to the *cbh1* signal sequence yielded enzymatically active xylanase protein at a level of 0.5 g / l (de Faria *et al.*, 2002). However, fusion of *xyn* to CBHI core-linker resulted in a considerably lower amount of xylanase.

A summary of the published reports on expression of cellulases in *T. reesei* is presented in Table 5. Production of cellulases in *T. reesei* has been performed for various reasons. To improve the production of *T. reesei* EGI in a hypercellulolytic mutant strain, the *egl1* promoter of *T. reesei* was exchanged with the *T. reesei cbh1* promoter and the copy number of the *egl1* gene was increased (Karhunen *et al.*, 1993). The *egl1* cDNA was expressed from the *cbh1* promoter as efficiently as *cbh1* itself. Furthermore, a strain carrying two copies of the *cbh1-egl1* expression cassette produced twice as much EGI as the amount of CBHI produced by the host strain. The level of *egl1*-specific mRNA in the single-copy transformant was about 10-fold higher than that found in the non-transformed host strain, indicating that the *cbh1* promoter is about 10 times stronger than the *egl1* promoter. This apparent discrepancy with the result of 3 times stronger reported by Ilmén *et al.* (1997) could be simply due to the use of different strains and different culture conditions. *T. reesei* EGIII has been overproduced for textile processing. The *egl3* gene product was expressed as the major secreted product (deduced from SDS-PAGE) of *T. reesei* by overexpressing it under the *cbh1* promoter in a quad deletion strain (Δ*cbh1*, Δ*cbh2*, Δ*egl1* and Δ*egl2*) (Bower *et al.*, 1998b). EGIII was produced as glycosylated and unglycosylated enzymes. There was no apparent difference in thermostability, pH stability or in spesific activity of the two glycoforms.

T. reesei cellulases have also been expressed in *T. reesei* as fusions to different tags (purification aids). Karlsson *et al.* (2001) performed homologous expression of *T. reesei* EGIV (Cel61A) as a fusion protein with a histidine tag under the *cbh1* promoter in order to facilitate the purification of EGIV from the other cellulases. The tagged protein was produced in significant amounts (yield at the g / l level not reported) and 120 mg EGIV could be purified from 1 liter of culture filtrate. Collen *et al.* (2002) investigated the effects of fusion tags on partitioning of EGI to the hydrophobic detergent-enriched phase of an aqueous two-phase system to facilitate purification of cellulases. *T. reesei* EGI core fused with a peptide molecule $(WP)_4$ (= Trp-Pro)$_4$ was expressed under the *cbh1* promoter in *T. reesei*. Production of the fusion protein was lower than that of the endogeneous EGI production and only approximately 15 % of the total EG activity corresponded to the fusion protein $EGI_{core\text{-}P5}(WP)_4$.

Because the *cbh1* promoter is repressed by glucose, promoters active in glucose-containing media have also been screened and tested for cellulase production. A synthetic glucose-containing medium would be beneficial, because it is expected to support synthesis of lower levels of proteases, a condition important to production, and it would allow easier purification of the product (Nakari-Setälä and Penttilä, 1995). Some genes, such as *pgk1* encoding phosphoglycerate kinase and *pki1* encoding pyruvate kinase, active in the presence of glucose have been isolated, but only modest cellulase levels have been obtained by using these promoters (reviewed in Penttilä, 1998). *T. reesei cbh1* and *egl1* cores have been expressed by the promoters of the elongation factor 1α, *tef1*, and the unidentified gene for cDNA1 (Nakari-Setälä and Penttilä, 1995). In glucose-containing medium the cDNA1 promoter gave the highest amounts of CBHI and EGI core, more than 50 mg/l, and accounted for more than half of the total protein secreted by the fungus. Although the expression levels were rather high, they were not comparable to the endogenous cellulase protein levels produced on cellulase-inducing media. Furthermore the amount of total secreted proteins remained low (0.05–0.23 g/l), compared with that obtained with this strain on cellulose medium (1.1–1.8 g/l).

Recently a new promoter (*hex1* gene encoding hexagonal protein of the fungal Woronin body) of *T. reesei* has been described with option for gene expression (Nevalainen *et al.*, 2003). The *hex1* promoter was shown to be highly functional in both glucose medium and under conditions promoting induction of *cbh1*. *Hex1* promoter with a secretion signal sequence was used to express heterologous DsRed1-E1 -reporter gene and DsRed1-E1 message was observed (the amounts of DsRed1-E1 message produced were not reported).

Table 5. Expression of fungal cellulases in T. reesei.

Promoter	Cellulase gene	*T. reesei* host strain	Amount	Reference
Cellulase promoters				
T.r. cbh1	*T.r. egl1*	VTT-D-79125	EGI 1.9 g/l	Harkki *et al.*, 1991
T.r. egl1	*T.r. egl1*	VTT-D-79125	EGI 2.2 g/l, 3x parent	Karhunen *et al.*, 1993
T.r. cbh1	*T.r. cel61A* - His tag fusion	Rut-C30	Not determined	Karlsson *et al.*, 2001
T.r. cbh1	*T.r. egl1*$_{\text{core-P5}}$(WP)$_4$ fusion	Rut-C30	< endogeneous EGI	Collen *et al.*, 2002
T.r. cbh1	*T.r. egl3*	RL-P37	Major secreted product	Bower *et al.*, 1997, 1998b
T.r. endogeneous *cbh1*	*T.r.* endogeneous *cbh1*	*T.r.* strain overproducing *S. cerevisiae* MPD synthase	Amount of secreted proteins 7x wild type, elevated CBHI	Kruszewska *et al.*, 1999
T.r. cbh2	*T.r. cbh2*	QM9414	CBHII 34 µg / l, 2-4x parent	Kubicek-Pranz *et al.*, 1991
Promoters active in the presence of glucose				
T.r. modified *cbh1*	*T.r. egl1* core	QM9414	EGI 2 mg/l	Nakari-Setälä *et al.*, 1993
T.r. pgk1	*T. r. egl1*	QM9414	EG activity below detection limit, no result with Mab EGI	Vanhanen, 1991
T.r. cDNA1	*T. r. egl1 and cbh1* core	QM9414	EGI and CBHI 50 mg/l	Nakari-Setälä *et al.*, 1995
T.r. tef1	*T. r. egl1* core	QM9414	EGI 2 mg/l	Nakari-Setälä *et al.*, 1995

T.r. = *T. reesei.* The results are from shake flask cultivations.

1.3.3 Secretory pathway

The molecular mechanism of the protein secretion system of filamentous fungi, including *T. reesei*, has recently been studied to an increasing extent in order to further improve the efficiency of protein secretion (Radzio and Kück, 1997, Veldhuisen *et al.*, 1997, Conesa *et al.*, 2001, Saloheimo *et al.* 2004). In fungi, protein folding, glycosylation, disulphide bridge formation, phosphorylation and subunit assembly are performed in the endoplasmic reticulum (ER). ER-related events contribute to the folding of proteins and are believed to be one of the main reasons for low yields of heterologous products (Penttilä *et al.*, 2004). The unfolded protein response (UPR) mechanism detects the presence of unfolded proteins in the ER and induces the synthesis of folding enzymes (reviewed in Conesa *et al.*, 2001). ER-resident chaperones and foldases assist in protein secretion.

The gene *pdi1* encoding protein disulphide isomerase, an ER foldase, has been isolated from *T. reesei* (Saloheimo *et al.*, 1999). The *pdi1* promoter has two potential UPR elements and it was shown that the gene is under the control of the UPR pathway. A *T. reesei hac1* gene encoding the UPR transcriptional factor has been described (Saloheimo *et al.*, 2003a). Saloheimo *et al.* (2003b) observed that concurrently with the induction of the UPR pathway, the genes encoding secreted proteins are rapidly down-regulated in *T. reesei*. This type of regulation can be caused by different secretion inhibitors and by foreign protein expression.

In eukaryotes proteins travel from ER to the Golgi apparatus, where additional modifications (glycosylation) take place. The structure and function of secreted proteins can be modified by glycosylation processes. In general, filamentous fungi produce high mannose type N-glycans and are also capable of effective O-glycosylation (reviewed in Nevalainen, 2001). O-glycosylation has been shown to be essential for secretion of EGI and EGII in *T. reesei* (Kubicek *et al.*, 1987). Kruszewska *et al.* (1999) overexpressed in *T. reesei* a gene encoding *Saccharomyces cerevisiae* mannosylphosphodolichol synthase, which is required for glycan synthesis and is a key enzyme in the O-mannosylation reaction, and were able to increase the level of secreted CBHI (Table 5). This suggests that insufficient glycosylation might limit the overproduction of glycoproteins.

Finally, proteins packed in secretory vesicles are directed to the plasma membrane from where they are secreted. There is evidence that the secretory pathway also involves transport from the ER through the Golgi complex to the plasma membrane in *T. reesei* (Kruszewska *et al.*, 1999). Most studies indicate that protein secretion in filamentous fungi occurs in the apical or subapical hyphal regions (Archer and Peberdy, 1997). However, the *cbh1* mRNA and CBHI of *T. reesei* were both found to localize to all hyphae of a colony (Nykänen *et al.*, 1997). Thus *cbh1* mRNA can be found not only in apical compartments involved in the growth of the hyphae, but also in the old compartments of the colony.

1.4 Cellulases in cotton finishing

Cotton is the most important of all textile fibres. In 2001 about 21 Mt of cotton was produced, which accounts for about 32 % of the world textile fibre production (CIRFS, 2002). Processing of cotton fabric includes preparation, dyeing, printing and finishing (Fig. 4). The purpose of cotton preparation is to remove impurities and prepare the fabric for dyeing and for any other wet processing treatments that follow, such as printing and finishing (Rouette, 2002). Different processing steps are included in cotton preparation: desizing (removing sizing agents), scouring (removing impurities such as pectins and waxes) and bleaching (increasing whiteness). The textile finishing step provides a method whereby deficiences in the textile can be corrected or specific properties can be introduced. The most important cellulosic finishes include crease resistant and stabilizing finishes, soil release and softening finishes, oil and water repellant finishes, biologically protective finishes, and flame retardant finishes (Needles, 1986). Cellulase enzymes have been used as finishing agents in processing of cotton-containing materials since the 1980s (Oslon and Stanley, 1990). Cellulase treatments are carried out in the textile wet processing stage mainly before or after dyeing.

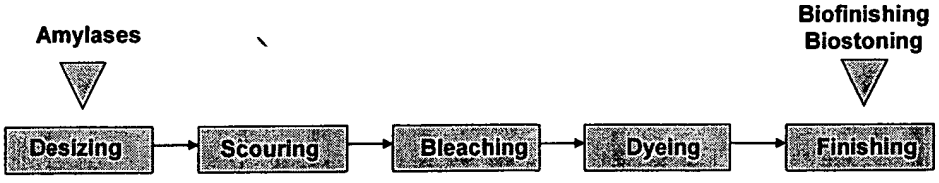


Figure 4. General processing stages of cotton fabric.

1.4.1 Use of cellulases in denim finishing

Denim is cotton twill-weave fabric with a dyed warp and raw white weft. The warp yarn is traditionally dyed with blue indigo. Stone-washed finish of denim refers to indigo-dyed denim with a faded appearance (Rouette, 2002). This is traditionally achieved by using pumice-stones when washing the desized (starch coating removed) articles, so that the dye is washed out partially and unevenly from the fabric. Cellulases have been introduced in the finishing of denim jeans and other denim garments to replace pumice stones and to achieve a washed-out appearance similar to that provided by pumice stones (Olson and Stanley, 1990). The process is called biostoning (Fig. 4). Cellulases attack primarily the surface of the fibre, but leave its interior intact. This mode of action makes cellulases especially suitable for stonewashing of denim garments dyed with indigo-blue, as the dye is located on the surface of the fibre (Tyndall, 1990). Washing the garments with stones partially removes this surface dye by abrasion. Cellulase treatment results in similar effect, as cellulases partially hydrolyze the surface of the fibre including the dye (Kochavi *et al.* 1990). In both cases mechanical action is needed to remove the dye and thus biostoning is usually carried out in e.g. jets or rotating drum washers. There are several advantages of using cellulases instead of stones. The use of cellulases prevents the damage both to the washing machines and to the garments, eliminates the need for disposal of the used stones, improves the quality of the waste-water and eliminates the need for labour-intensive removal of dust from the finished garments (Kochavi *et al.*, 1990). Furthermore the garment load can be increased by as much as 50 % as no stones need to be added to the machine.

Different types of cellulases are used for biostoning. The enzymes differ in a number of ways, one of the differences being pH optimum. Cellulases used for biostoning have traditionally been classified by the pH optimum of the enzyme: neutral cellulases operate in the pH range 6–8, alkaline-neutral cellulases in the pH range 7.5–8 and acid cellulases in the range of pH 4.5–6 (Videbaek *et al.*, 1994, Klahorst *et al.*, 1994). Acid cellulases used in biostoning mainly originate from *T. reesei* and are characterized by highly aggressive action on cotton, resulting in abrasion in a short time and associated with backstaining. Neutral and alkaline cellulases used in biostoning come from a variety of fungi (Table 6) and generally have been characterized by less aggressive action on cotton than

acid cellulases, requiring a longer wash time and resulting in little or no backstaining (Klahorst *et al.*, 1994, Solovjeva *et al*, 1998).

Cellulase preparations from various sources differ in their denim-washing performance and backstaining (Gusakov *et al.*, 1998, 2000a). Gusakov *et al.* (2000a) showed that commercial and laboratory cellulase preparations produced by different fungi (from the genera *Trichoderma, Penicillium, Chaetomium, Humicola,* and others) differed in their abrasive effect (the ability to remove indigo from denim) and no direct correlation was found between the ability to remove indigo and any specific cellulase activity (CMC, endoglucanase, filter paper activity, avicelase). Gusakov *et al.* (2000b) indicated that there is a certain correlation between the washing performance and the quantity of non-polar amino acid residues in the enzyme. Heikinheimo *et al.* (2000) indicated that by selecting the optimum *T. reesei* cellulase profile, the cellulase action can be directed towards the indigo-dyed parts, and further that endoglucanases are the cellulases required for good stone washing effects.

1.4.1.1 Backstaining

During biostoning with cellulases the released indigo dye has a tendency to redeposit on the surface of the denim fabric, thus reducing the desired contrast between white and the indigo-dyed blue yarn. This effect is termed backstaining and it is undesired because of lowered contrast between the blue and white yarn. In early reports backstaining was claimed to be dependent on pH (Kochavi *et al.*, 1990). Further experiments indicated that the nature of the enzyme used has an impact on backstaining, and neutral cellulases were found to cause low backstaining (Klahorst *et al.*, 1994). Indigo-cellulase affinities and enzyme adsorption on the white yarn of the denim fabric have been shown to cause backstaining (Cavaco-Paulo *et al.*, 1998b, Gusakov *et al.*, 1998, 2000b). *T. reesei* cellulases adsorbed onto cotton fabric caused higher indigo staining than comparable amounts of adsorbed *H. insolens* neutral cellulases (Cavaco-Paulo *et al.*, 1998b). Adsorption studies with plain indigo showed that cellulases from *T. reesei* with a higher content of neutral amino acids have a higher affinity for indigo dye than cellulases of *H. insolens* (Campos *et al.*, 2000). The authors also proposed that the non-polar residues present in higher percentages in the cellulases of *H. insolens* play an important role in the agglomeration of indigo

dye particles and probably in the reduction of backstaining. Gusakov *et al.* (1998) showed by using a model microassay system that backstaining by different enzyme preparations is different and suggested that protein adsorption on cotton garment is a crucial parameter causing backstaining. Gusakov *et al.* (2000b) showed with immobilized amino acids that indigo may be bound to non-polar amino acids.

Backstaining can be avoided by adding antiredeposition chemicals during the enzyme washing step and/or by adding a mild bleaching agent or stain removing agents during the rinsing steps (Tyndall, 1990, Yoon *et al.*, 2000). The use of different, particularly *Trichoderma,* cellulase compositions with less specific activity on denim has also been tested (Clarkson *et al.*, 1994a). Use of truncated *T. reesei* EGI and EGII cellulases (EGI and EGII catalytic core) and truncated CBHI (core) with EGIII was found to decrease backstaining while maintaining an equivalent or superior level of abrasion over their un-modified counterparts (Fowler *et al.*, 2001). Protease enzyme added during rinsing or at the end of the cellulase washing step resulted in significant reduction of backstaining and improved contrast (Yoon *et al.*, 2000). Denim that was stonewashed with the addition of lipolytic enzyme during cellulase treatment showed a reduction in the level of backstaining, especially the backstaining of pocket parts (Uyama and Daimon, 2002).

1.4.1.2 Effect of different cellulases in biostoning

The origins of the fungal cellulases used in biostoning and their reported effects are summarized in Table 6. In addition to the examples in Table 6, the use of *T. reesei* cellulases in biostoning has been described in numerous other publications. Cellulases of *T. reesei* are widely used in denim finishing one reason being their low price. Furthermore although *Trichoderma* cellulases result in high backstaining they are preferred because of their high activity on denim material, resulting in a short processing time (Clarkson *et al.*, 1994a). Finishing of denim with different ratios of *T. reesei* EGI and II and CBHI and II has been reported in several publications (Clarkson *et al.*, 1992a, 1992b, 1993, 1994a, b). Heikinheimo *et al.* (2000) showed that purified *T. reesei* EGII is the most effective cellulase for removing colour from denim, producing a good stone washing effect with the lowest hydrolysis level.

Schülein *et al.* (1998) isolated family 45 cellulases from six different fungal sources (Table 6) and found that they could be used for increased abrasion in denim finishing due to their efficient removal of indigo from the surface of cotton. They also isolated three family 7 cellulases from different sources (Table 6) and these cellulases accomplished very little abrasion. Murashima *et al.* (2000) isolated a novel cellulase NCE4 from *Humicola insolens* that can be used for various treatments of cellulose-containing fibres, such as for decolouring denim-dyed cellulose-containing fibres. A cellulase preparation of *Chrysosporium lucknowense* had higher abrasive activity and lower backstaining on denim than the *T. reesei* preparation, when a model microassay was used for testing denim-washing performance (Sinitsyn *et al.,* 2001). Of the four individual cellulase components (two endoglucanases – EG-25 and EG-50, and two cellobiohydrolases – CBH-43 and CBH-55) of *C. lucknowense*, EG-25 was the key enzyme responsible for indigo removal with relatively low backstaining. A cellulase preparation of *Penicillium occitanis* was successfully applied in a biostoning process in industrial scale (Belghith *et al.,* 2001). Cellulases (EGI) derived from *Myceliophthora thermophila* exhibited enhanced enzyme activity in the alkaline pH range and are described to be useful in preventing backstaining, but no examples of denim washing were presented in the patent publication (Osten and Schülein, 1999). In addition to fungal enzymes, bacterial and actinomycete cellulases have also been applied in denim treatment (van Beckhoven *et al.*, 1996, Farrington *et al.,* 2001, van Solingen *et al.*, 2001).

Table 6. Fungal cellulases in biostoning applications.

Source	Cellulase	Application pH	Special effect	Reference
T. reesei	No or low CBHI EG:CBH, 5:1 Enriched CBHI EGII (purified) EGIII EGIII-dimer	4.5–5.5	Low strength loss Lint reduction, low strength loss Decreased strength loss Low hydrolysis level Suggestion of reduced strength loss in textile processing	Clarkson *et al.*, 1992a,b Clarkson *et al.*, 1992c, 1994b Clarkson *et al.*, 1993 Heikinheimo and Buchert, 2001 Fowler *et al.*, 2001 Bower *et al.*, 1998b
T. terrestris	EG (299 aa)	5	Almost bleached appearance	Schülein *et al.*, 1996
Acremonium sp.	EG (295 aa)	7	Low temperature optimum, high abrasion level	Schülein *et al.*, 1996
M. thermophila	EG (225 aa) + linker and CBM of *H. insolens* 43 kDa EG	6	Minimum strength loss, high abrasion level	Schülein *et al.*, 1996
H. insolens, M. thermophila, Acremonium sp., *M. phaseolina, T. terrestris, C. scabela*	EGV (Cel45)	n.r.	Good abrasion, fuzz removal	Schülein *et al.*, 1998
H. insolens, F. oxysporium	EGI (Cel7)	n.r.	Low strength loss, little abrasion	Schülein *et al.*, 1998
M. thermophila	EGI and variants	n.r.	Enhanced activity in the alkaline pH range	Osten and Schülein, 1999
H. insolens	EGV + EGI	7	Combination of desizing and stonewashing: abrading and streak-reducing	Lund, 1997
H. insolens	NCE4	7	De-colouring of denim	Murashima *et al.*, 2000
C. lucknowense	Total mixture	6.5 - 8	Similar wash performance to commercial neutral cellulase (Denimax XT / Ultra)	Solovjeva *et al.*, 1998
C. lucknowense	Total mixture EG-25	5	High abrasion on denim, prevention of backstaining	Sinitsyn *et al.*, 2001
P. occitanis		5.5	Denim finishing	Belghith *et al.*, 2001

1.4.2 Use of cellulases in biofinishing

Cellulase treatment for finishing of cellulose-containing textile materials, such as cotton, linen, lyocell and viscose materials, is called biofinishing or biopolishing (Videbaek and Andersen, 1993, Fig. 4). In the biofinishing process, cellulases achieve a controlled surface hydrolysis and the fibre ends protruding from the fabric surface are weakened and subsequently separated from the material with the aid of mechanical action. The benefits of cellulase treatment of cotton fabrics and garments include permanent improvement of depilling, cleared surface structure by reduced fuzz, improved textile softness, improved drapeability, brighter colours of the textile, improved dimensional stability and fashionable wash-down effects (Tyndall, 1992, Pedersen *et al.,* 1992, Kumar *et al.,* 1997, Cavaco-Paulo, 2001, Cortez *et al.,* 2002). One advantage of the biofinishing process is that the treatments can be adapted to run on existing equipment in the textile industry. In most cases the treatments have been carried out on garments and fabrics. Treatment of cotton yarn with a *Trichoderma* EGII was reported to result in decreased hairiness and increased evenness (Pere *et al.,* 2001). When the treated yarn was further knitted to a fabric, a decreased tendency to pilling was observed. Yarn treatment for pilling control may offer an advantage by overcoming the dust problems often encountered with biofinishing of knitted fabrics.

Of the *T. reesei* cellulases the endoglucanases have been shown to play a key role in biofinishing. Several studies have been conducted to evaluate the best cellulase component or cellulase combination for biofinishing with minimal effects on the weight and strength properties of the fabric. Heikinheimo *et al.* (1998) showed that purified *T. reesei* EGI and II caused more strength loss than purified CBHI, but also had positive effects on bending behaviour and pilling properties of cotton twill and poplin fabrics. According to the results of Heikinheimo and Buchert (2001) there are clear differences between individual purified CBHI, CBHII, EGI and EGII and their defined mixtures. Treatment of cotton interlock fabric showed that EGII-based combinations always resulted in good depilling properties. Furthermore, with an EGII: CBHI ratio of 25:75, practically no decrease in strength was observed despite high depilling. Pure CBHI or CBHII did not affect pilling properties, whereas EGI and EGII treatments clearly improved pilling values. Relatively more EGI was required as compared to EGII in order to obtain a similar improvement in pilling resistance.

T. reesei EGIV (Cel61A) has been included in experiments searching for defibrillating activity together with other several *T. reesei* cellulases, but no defibrillating activity was found (Karlsson *et al.*, 2001).

With experimental *T. reesei* cellulase preparations, it was found that high pilling removal is dependent on the fabric type (Miettinen-Oinonen *et al.*, 2001). In all cases, EGII-based cellulase products gave the most positive depilling result. On the other hand removal or decrease of EGII in a cellulase composition resulted in better strength properties of cellulose-containing textile materials as compared with treatment with the whole cellulase mixture of *T. reesei* (Miettinen-Oinonen *et al.*, 1996). Clarkson *et al.* (1993, 1992b) described the use of either CBHI-enriched *T. reesei* or a preparation free of CBHI to obtain reduced strength loss of the fabrics. By using an EG:CBH composition of 5:1 improved feel and appearance was obtained with low strength loss and without production of an excessive amount of lint (Clarkson *et al.*, 1992c, 1994b). Kumar *et al.* (1997) tested *T. reesei* whole cellulase (complete with endo- and exoactivities) and an endo-enriched product, enriched with specific EG components (more precise information on the EG was not given) and with partial removal of CBH components. Whole acid cellulase was found to be best for cotton when a high level of surface polishing was required. The endo-enriched cellulase was useful as a less aggressive cellulase that can help minimize strength loss. According to Lenting and Warmoeskerken (2001) the use of a single type of cellulase can avoid substantial breakdown of crystalline cellulose and therefore minimize tensile strength loss.

Liu *et al.* (2000) investigated three cellulase compositions for the biofinishing of cotton interlock knitted fabric. An acid cellulase complex, an endo-enriched cellulase and a mono-component endoglucanase (the origin of the preparations was not given), which all had similar application pH and temperature ranges, were shown to have different sensitivities to liquor ratio and to mechanical agitation. The effects of process conditions were also studied by Cavaco-Paulo *et al.* (1998a) and Cortez *et al.* (2001), who showed that increasing mechanical agitation favours attack by *Trichoderma* EG-rich cellulase product as compared to CBH-rich or total crude mixture. According to Liu *et al.* (2000) several parameters affect successful biofinishing: pH, temperature, liquor ratio, enzyme concentration, time, mechanical agitation, fabric type and product quality,

cellulase characteristics such as selectivity depending on the machine type, custom needs, and cellulase composition.

In addition to *Trichoderma* cellulases, cellulases originating from e.g. *Humicola insolens* and *Cellulomonas fimi* have been used in treatments of cotton (Lund and Pedersen, 1996, Boisset *et al.*, 1997, Azevedo *et al.*, 2000).

1.5 Aims of the present study

The general aim of this study was to construct *T. reesei* cellulase production strains for industrial use for various applications and especially for applications in the textile industry.

The specific goals were:

- to construct different genetically tailored strains producing high levels of *T. reesei* endoglucanase and cellobiohydrolase for specific applications,
- to study the use of the preparations derived from the tailored *T. reesei* strains in biostoning and biofinishing of cotton,
- to identify novel cellulases with cellulolytic activity over a broad pH range, especially at neutral pH, and functioning in biostoning with low backstaining,
- to isolate novel genes encoding the neutral cellulases and transfer them to *T. reesei* for effective production of the cellulases for use in biostoning at neutral pH.

2. Materials and methods

A summary of the materials and methods used in this work is presented in this section. Those described in sections 2.8.1 and 2.8.2 were also published in US Patent 5,874,293. More detailed information is given in the original publications I–IV.

2.1 Strains and plasmids

Escherichia coli strain XL1-Blue was used for propagation of plasmids. pUC19 was used as a vector backbone in plasmid constructions. Plasmids constructed for expression of *T. reesei* and *M. albomyces* cellulases in *T. reesei* are listed in Table 7.

Table 7. Properties of DNA fragments used for transformation of T. reesei for expression of T. reesei (Tr) or M. albomyces (Ma) cellulases.

Plasmid	*T. reesei* promoter / terminator	Expressed gene	Flanking regions*	Marker	Reference
pALK537	*cbh1/ cbh1*	*Tr egl2*	*cbh1*	*amdS*	I
pALK496	*cbh1/ cbh1*	*Tr cbh1*	*egl1*	*amdS*	II
pALK540	*cbh1/ cbh1*	*Tr egl2*	*cbh2*	*ble*	I
pALK543	*cbh2/ cbh2*	*Tr cbh2*	*egl2*	*ble*	II
pLAK546	*cbh1/ cbh1*	*Tr cbh2*	*egl2*	*ble*	II
pALK1231	*cbh1/ cbh1*	*Ma cel45a*	*cbh1*	*amdS*	IV
pALK1235	*cbh1/ cbh1*	*Ma cel45a*	*egl1*	*hygB*	IV
pALK1238	*cbh1/ cbh1*	*Ma cel7a*	*cbh1*	*amdS*	IV
pALK1240	*cbh1/ cbh1*	*Ma cel7a*	*egl1*	*hygB*	IV
pALK1242	*cbh1/ cbh1*	*Ma cel7b*	*cbh1*	*amdS*	IV

*5' and 3' regions of the *T. reesei* cellulase gene used to target the expression cassette in the corresponding locus.

Melanocarpus albomyces ALKO4237, *Myceliophthora thermophila* ALKO4179, *Chaetomium thermophilum* ALKO4265 and *Sporotrichum thermophilum* ALKO4125 were used for production of cellulases. A summary of the cellulase compositions of the *T. reesei* strains used and constructed in this work is presented in Table 8.

Table 8. Cellulase compositions of the T. reesei strains constructed and used in this work.

Strain	Cellulase composition*		Reference
	T. reesei cellulases**	*M. albomyces* cellulases	
VTT D-79125	CBHI, CBHII, EGI, EGII (hypercellulolytic mutant)	-	Bailey and Nevalainen, 1981
ALKO2221	CBHI, CBHII, EGI, EGII (low protease mutant of VTT D-79125)	-	Mäntylä *et al.*, 1994
ALKO3760	**CBHI+**, CBHII, EGI, EGII	-	II
ALKO3862	**CBHI+**, CBHII, EGI, EGII	-	II
ALKO3761	**CBHI+**, CBHII, EGI-, EGII	-	II
ALKO3798	CBHI, **CBHII+**, EGI, EGII	-	II
ALKO3799	CBHI, **CBHII+**, EGI, EGII	-	II
ALKO3873	CBHI, **CBHII+**, EGI, EGII-	-	II
ALKO4095	**CBHI+**, **CBHII+**, EGI-, EGII- (*cbh1* promoter for *cbh2* expression)	-	II
ALKO4097	**CBHI+**, **CBHII+**, EGI-, EGII- (*cbh2* promoter for *cbh2* expression)	-	II
ALKO2698	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.*, 1993
ALKO2697	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.*, 1993
ALKO2656	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.*, 1993
ALKO3529	CBHI, CBHII, EGI, **EGII+**	-	I
ALKO3530	CBHI-, CBHII, EGI, **EGII+**	-	I
ALKO3574	CBHI-, CBHII, EGI, **EGII+**	-	I
ALKO3528	CBHI-, CBHII-, **EGI+**, **EGII+**	-	I
ALKO3620	CBHI, CBHII, EGI, EGII-	-	Suominen *et al.*, 1993
ALKO4072	CBHI-, CBHII, EGI, EGII-	-	M. Paloheimo, Roal Oy, Finland
A3620/1231/14 and 16	CBHI-, CBHII, EGI, EGII-	**Cel45A+ (EGV+)**	IV
A3620/1235/40 and 49	CBHI, CBHII, EGI-, EGII-	**Cel45A+ (EGV+)**	IV
A3620/1238/42	CBHI-, CBHII, EGI, EGII-	**Cel7A+ (EGI+)**	IV
A3620/1240/32	CBHI, CBHII, EGI-, EGII-	**Cel7A+ (EGI+)**	IV
A3620/1242/13	CBHI-, CBHII, EGI, EGII-	**Cel7B+ (CBHI+)**	IV

* Overproduction is indicated as + **and in bold** and deletion as - and in gray.

** Listing main cellulase components.

2.2 Media, growth of organims and transformation of *Trichoderma*

Media and cultivation conditions are described in detail in articles I–IV. Transformation of *T. reesei* was carried out as described by Penttilä *et al.* (1987) with the modifications described by Karhunen *et al.* (1993).

2.3 DNA techniques

Standard DNA techniques as described by Sambrook *et al.* (1989) and Maniatis *et al.* (1982) were used. Construction and screening of an *M. albomyces* genomic library, isolation of plasmid and chromosomal DNA, isolation of DNA fragments for cloning, sequencing of DNA, amplification of DNA fragments and genes by PCR and Southern blot analysis are described in detail in articles I-II and IV. The primers used to amplify the genes encoding *M. albomyces* 20 and 50 kDa EGs and 50 kDa CBH are shown in Table 1/IV.

2.4 Enzyme activity assays

Endoglucanase activity was measured at pH 4.8 as the release of reducing sugars from hydroxyethyl cellulose (HEC, ECU-activity) as described by Bailey and Nevalainen (1981). At neutral pH endoglucanase activity was measured using HEC or carboxymethyl cellulose (CMC) as substrate by the same method with the following modifications: 1 % HEC was used at 50°C, pH 7 or 3 % CMC at 50 °C or 70 °C (NCU activity), pH 7 in 50 mM Hepes buffer. Activity against barley β-glucan was assayed in the same way as activity against HEC (Bailey and Nevalainen, 1981), replacing HEC by barley β-glucan. Filter paper-hydrolyzing activity (FPU) was measured according to the method by Mandels *et al.* (1976). Cellobiohydrolase I activity was measured as activity against 4-methylumbelliferyl-β-D-lactoside (MUL) according to van Tilbeurgh *et al.* (1988). The total MUL activity represents activities of EGI and CBHI. The MUL (CBHI) activity was measured by inhibiting CBHI in the presence of 5 mM cellobiose and by subtracting the MUL (EGI) activity thus obtained from the total MUL activity. β-Glucosidase activity was measured using 4-nitrophenyl-β-D-glucopyranoside as substrate as described in Bailey and Nevalainen (1981).

2.5 Purification of *M. albomyces* cellulases

Purification of the 20 and 50 kDa EGs and the 50 kDa CBH from *M. albomyces* ALKO4237 is described in article III. Digestion of the purified proteins and separation of the peptides as well as the subsequent protein and peptide sequencing are explained in article III.

2.6 Immunological methods

The presence of the *Trichoderma* EGI, CBHI and CBHII proteins was detected from Western blot filters (Towbin *et al.*, 1979) or from Dot blot filters (Schleicher & Schüll) immunostained using a monoclonal EGI antibody EI-2, CBHI-89 antibody and CBHII antibodies CII-8 and CII-30 (Aho *et al.*, 1991) and the Protoplot Western Blot AP system (Promega). *Melanocarpus* Cel45A, Cel7A and Cel7B-specific polyclonal antibodies were used in a similar manner for detection of the respective proteins. Quantitation of secreted *T. reesei* EGI, CBHI and CBHII was carried out by a double antibody sandwich ELISA (Bühler, 1991) using the monoclonal antibodies EGI-2, CI-258 and CII-8 (Aho *et al.*, 1991) as capture antibodies.

2.7 Biofinishing

Biofinishing of cotton fabric was performed at pH 5 with cellulase preparations of *T. reesei* VTT D-79125, ALKO3529, ALKO2656, ALKO3528, ALKO3760, ALKO3798 and ALKO4097 (Table 8) and the effects were evaluated visually and by determining pilling and weight loss as described in detail in article II.

2.8 Biostoning

Biostoning of denim fabric was performed as described in articles I, III–IV and in sections 2.8.1 and 2.8.2. Cellulase preparations of *T. reesei* VTT D-79125, ALKO3529, ALKO3528, ALKO2656, ALKO3760, ALKO3798, ALKO4097 (Table 8) and Ecostone L (Primalco Ltd. Biotec) were used in biostoning at pH 5. At pH 7 culture supernatants of *M. albomyces* ALKO4237, *M. thermophila* ALKO4179, *S. thermophilum* ALKO4125 and *C. thermophilum* ALKO4265,

purified *M. albomyces* Cel45A, Cel7A and Cel7B cellulases and *T. reesei* recombinant *Ma* Cel45A cellulase preparations were used.

2.8.1 Biostoning with purified *T. reesei* cellulases

Denim fabric was prewashed for 10 min at 60°C with an amylase product Ecostone A 200 (1ml/l, Primalco Ltd. Biotec, Finland). The fabric was cut into 12x12 cm swatches. The colour was measured from the fabric as reflectance values with the Minolta (Osaka, Japan) Chroma Meter 1000 R L*a*b* system. Cellulase treatments were performed in an LP-2 Launder-Ometer (Atlas, USA). About 7 g of denim swatches were loaded into the 1.2 litre container containing 200 ml of 50 mM citrate buffer, pH 5. 10 steel balls were added into each container to help the colour removal. *T. reesei* preparation produced using the hypercellulolytic mutant strain VTT D-79125 (Table 8) and cultivated as in article I was used. 300 ECU per g of fabric was used in each test and purified *T. reesei* CBHI, CBHII, EGI and EGII were added at dose levels of 1 or 2 mg per g of fabric. Cellulases were purified according to Pere *et al.* (1995) and Rahkamo *et al.* (1996) and were obtained from Matti Siika-aho (VTT Biotechnology, Finland). The containers were loaded into a 50 °C Launder-Ometer bath. The Launder-Ometer was run at 42 rpm for 1 or 2 hours. After removing the swatches from the containers they were soaked for 10 min in 200 ml of 10 mM NaOH and rinsed 2x5 min with cold water. The swatches were dried for 1 h at 105 °C and air dried overnight at room temperature. The colour from both sides of the swatches was measured with the Minolta Chroma Meter.

2.8.2 Biostoning with *T. reesei* cellulase preparations with enhanced CBH activity

Denim fabric was treated with cellulase preparations derived from the strains overproducing CBHI (ALKO3760), CBHII (ALKO3798) and both CBHI and CBHII (ALKO4097) and from VTT D-79125 (Table 8). The strains and cultivation conditions for obtaining the cellulase preparations are described in II. The experimental set-up was as in 2.8.1. 3 or 6 mg of the total protein (Lowry *et al.*, 1951) in the cellulase preparations per g of fabric was used in each experiment. The washing times were 1 and 2 hours at 50 °C.

3. Results

3.1 Construction of *T. reesei* strains overproducing EG and CBH (I, II)

Eight different types of *T. reesei* strains producing elevated amounts of homologous EGs or CBHs were constructed. The aim was to construct different tailored high endoglucanase activity or high cellobiohydrolase activity-producing strains for specific industrial applications, *i.e.* for the textile industry. The production of *T. reesei* EGII, CBHI and CBHII was improved in separate strains. Strains producing high levels of EGI and EGII without any cellobiohydrolases (EG-overproducing strains) or CBHI and CBHII without the main endoglucanases EGI and EGII (CBH-overproducing strains) were also constructed. For overexpression of the *egl2, cbh1* or *cbh2* genes in *T. reesei* the powerful promoter of the *cbh1* gene of *T. reesei* was used. The *cbh2* promoter of *T. reesei* was also used in the CBH-overproducing strain for expression of *cbh2.*

3.1.1 EGII- and EG-overproducing strains

Two different plasmids were constructed. The plasmid pALK537 (Fig. 1/I, Table 7) was constructed for expression of the *egl2* gene under the control of the *cbh1* promoter either in the locus of *cbh1* or elsewhere in the genome of *T. reesei* VTT D-79125 depending on the homologous or non-homologous recombination. The plasmid pALK540 (Fig. 2/I, Table 7) was constructed for expression of *egl2* from the *cbh1* promoter and for replacement of the *cbh2* locus of *T. reesei* ALKO2698, an EGI-overproducing CBHI-negative strain and containing one copy of the *egl1* expression cassette (Karhunen *et al.,* 1993). For construction of the plasmids the gene replacement strategy developed by Suominen *et al.* (1993) was used.

The transformants were cultivated on cellulase-inducing medium and were first screened by measuring endoglucanase activity from the culture medium. 61 % of the best endoglucanase producing pALK537-transformants were CBHI negative as analyzed by dot blotting and immunostaining. The transformants producing the highest endoglucanase activity in the culture medium, ALKO3529 (CBHI-

positive) and ALKO3530 and ALKO3574 (CBHI-negative) were analyzed in more detail. Southern hybridization data showed that in ALKO3574 one copy and in ALKO3530 two copies of the transformed pALK537 fragment had replaced the coding region of the *cbh1* gene (Figure 3/I). ALKO3529 contained two copies of the transformed fragment integrated into or close to the *cbh1* locus (Figure 4/I).

The frequency of targeting the pALK540-fragment into the *cbh2* locus was 71 %. Of the pALK540-transformants, ALKO3528 produced the highest endoglucanase activity and was shown by Southern hybridization to contain one full-length copy of the *egl2* expression cassette in the *cbh2* locus (Figure 6/I).

3.1.1.1 Enzyme production

All the transformants and the parent strains VTT D-79125 and ALKO2698 were grown in shake flasks on cellulase-inducing medium. The results of the measurements of cellulase activities and ELISA analysis from the culture medium are shown in Tables 2 and 3/I and in Table 9 (summary). In EGII-transformants one copy of the *egl2* expression cassette increased the endoglucanase and β-glucanase activities about twofold (ALKO3574) and two cassettes (ALKO3529, ALKO3530) about 3-fold compared to the parent strain VTT D-79125. Higher increases in both endoglucanase and β-glucanase activities were detected in the EGII-overproducing strains compared to EGI-overproducing strains (Karhunen *et al.*, 1993), which were used as controls (Table 9). Endoglucanase activity was increased about twofold in the EG-overproducing transformant strain ALKO3528 compared to the parent strain ALKO2698 and fourfold compared to VTT D-79125, the parent of ALKO2698. FPU activity was decreased 60–70 % in the EGII-overproducing strains lacking the *cbh1* gene (ALKO3530, ALKO3574). EGII appears to affect the FPU-activity, since FPU of the CBHI-positive EGII-overproducing strain ALKO3529 was about 10 % higher than that of the parent strain. ALKO3528 does not contain *cbh1* or *cbh2* and thus its FPU-activity was decreased almost to zero.

As compared to the parent strain the amount of secreted EGII protein (evaluated visually in several SDS-PAGE analyses with different dilution series with a known concentration of purified EGII protein as a standard) was increased

2-fold with one expression cassette and 3.2-fold with two expression cassettes, being then approximately 1.3 g/l (Figure 5/I). Although the *cbh1* promoter was used in the *egl2* expression vector, the amount of secreted CBHI as analyzed by ELISA was not significantly changed in the CBHI-positive EGII-overproducing strain ALKO3529 compared to the parent strain (Table 2/I, Table 9). The production of both EGI and CBHII was increased in the EGII-overproducing strains and the lack of *cbh1* further increased the amounts.

Table 9. Summary of the properties and cellulase production levels of the EGII-, EGI- and EG-overproducing strains.

Strain	*cbh1/ cbh2**	*egl2* cassette copy no	*egl1* cassette copy no	HEC nkat/ml	β–glu-canase nkat/ml	FPU /ml	CBHI mg/ml	CBHII mg/ml	EGI mg/ml
VTT D-79125	+/+	0	0	1200	9300	5.3	3.7	0.05	0.36
ALKO3529	+/+	2	0	3400	23 000	6.0	3.4	0.07	0.59
ALKO3530	-/+	2	0	3600	25 000	2.1	na	0.08	0.59
ALKO3574	-/+	1	0	2800	19 400	1.7	na	na	0.65
ALKO2697	-/+	0	2**	2600	20 200	1.8	na	na	na
ALKO2698	-/+	0	1**	2300	15 700	1.6	na	na	na
VTT D-79125	+/+	0	0	1300	11 500	4.8	na	na	na
ALKO2698	-/+	0	1**	2600	15 300	1.4	na	na	na
ALKO3528	-/-	0	0	5100	27 800	0.2	na	na	na

*Southern analysis, **Karhunen *et al.* (1993), na = not analyzed.

3.1.2 CBHI-, CBHII- and CBH-overproducing strains

The plasmid pALK496 was constructed for inceasing the copy number of the *cbh1* gene in *T. reesei* and the plasmids pALK546 and pALK543 for expression of the *cbh2* gene from either the *cbh1* or the *cbh2* promoter (Figure 1/II, Table 7). The *T. reesei* strain ALKO2221 was used as a host in the transformations (Table 8).

T. reesei CBHI-overproducing transformants were screened by measuring the increased activity against 4-methylumbelliferyl-β–D-lactoside (MUL). 10 % of the transformants were EGI-negative as detected by Western blotting indicating replacement of the *egl1* gene by the expression cassette. Three transformants producing high MUL (CBHI) activity were analyzed in more detail. According

to Southern hybridizations the strain ALKO3761 contained one full-length copy of the *cbh1* expression cassette replacing the *egl1* gene (Figure 2/II). ALKO3862 and ALKO3760 contained two tandem copies of the *cbh1* expression cassette (in ALKO3760 the second copy had an incomplete pALK496 fragment) in unknown loci (not in *egl1* or *cbh1*) (Figure 2/II).

The *T. reesei* CBHII-overproducing transformants were screened for increased amounts of CBHII by the ELISA method (Bühler, 1991). The genomes of the transformant strains ALKO3873, ALKO3798 and ALKO3799 producing highest amounts of CBHII protein were analyzed by Southern blotting. The *egl2* gene was replaced by one copy of the pALK546 expression fragment in the transformant strain ALKO3873. ALKO3798 and ALKO3799 contained one copy of the transformed fragment integrated in an unknown locus (not *egl2, cbh2,* or *cbh1*). The 3'-end of the transformed fragment could not be detected in the Southern blots of ALKO3798 and ALKO3799 (Figure 3/II). However, on the basis of the ELISA analysis, SDS-PAGE and Western analysis these transformants produced additional complete CBHII and no truncated form of CBHII was detected (Table 2/II, Figure 4/II).

For construction of the strains overproducing both CBHI and CBHII without EGI and EGII (CBH-overproducing strains), the CBHI-overproducing *egl1*-negative strain ALKO3761 was transformed with the expression cassette from either the plasmid pALK543 or pALK546. The strains ALKO4095 (transformed with pALK546, *cbh2* under the *cbh1* promoter) and ALKO4097 (transformed with pALK543, *cbh2* under the *cbh2* promoter) had a single copy of the respective expression cassette replacing the *egl2* gene (Figure 5/II).

3.1.2.1 Enzyme production

The transformants and the host strains were cultivated in shake flasks on cellulase-inducing medium. The results of enzyme production are summarized in Table 10. The amount of EGI, CBHI and CBHII in the culture supernatants of the transformants were quantified by the ELISA method. In CBHI-overproducing transformants the amount of CBHI was increased 1.4–1.5 fold in the two-copy transformants ALKO3760 and ALKO3862 and 1.3-fold in the one-copy transformant ALKO3761 as compared to the host ALKO2221, resulting in

corresponding increase in MUL (CBHI) activity (Table 1/II, Table 10). The amount of CBHII was reduced by 25 % in ALKO3760 and ALKO3862, but increased by 15 % in ALKO3761. Although the *egl1* gene was intact in ALKO3760 and ALKO3862 the amount of EGI was decreased by 30–40 %, which was also detected as lowered endoglucanase activity. The level of total secreted proteins was increased in CBHI-overproducing transformants as compared to the host strain (Table 1/II).

ALKO3798 produced about four times and ALKO3799 and ALKO3873 about three times more CBHII than the host strain ALKO2221 (Table 2/II, Table 10). The amount of CBHI was decreased by 10–20 %. Production of endoglucanase activity was clearly decreased in the EGII-positive transformants ALKO3798 and ALKO3799. Production of endoglucanase activity was decreased by 50 % in the EGII-negative CBHII-overproducing strain ALKO3873.

Table 10. Summary of the properties and cellulase production levels of the CBHI-, CBHII- and CBH-overproducing strains.

Strain	*egl1/ egl2**	*cbh1* cassette copy no	*cbh2* cassette copy no	MUL** (CBHI)	FPU/ ml	HEC nkat/ml	CBHI mg/ml	CBHII mg/ml	EGI mg/ml
ALKO2221	+/+	0	0	1	3.6	700	2.2	0.18	0.32
ALKO3760	+/+	2	0	1.4	3.5	530	3.4	0.13	0.22
ALKO3862	+/+	2	0	1.3	3.4	490	3.1	0.13	0.17
ALKO3761	-/+	1	0	1.1	3.0	340	3.0	0.22	0.00
ALKO3798	+/+	0	1	na	3.6	570	1.7	0.70	0.31
ALKO3799	+/+	0	1	na	3.9	560	1.8	0.50	0.28
ALKO3873	+/-	0	1	na	3.0	360	1.9	0.53	0.39
ALKO2221	+/+	0	0	na	3.4	720	2.7	0.25	na
ALKO3761	-/+	1	0	na	2.8	390	3.9	0.23	na
ALKO4097	-/-	1	1	na	2.7	30	4.4	0.78	na
ALKO4095	-/-	1	1	na	2.5	30	3.5	0.85	na

* Southern analysis, ** ALKO2221 produced about 0.3 MUL (CBHI)/ml. The MUL (CBHI) activity of ALKO2221 was adjusted to 1 and the activities produced by the transformants are presented compared to the host strain, na = not analyzed.

Filter paper-hydrolyzing activity, which is mainly affected by cellobiohydrolases but also indicates total hydrolysis of cellulose, was not significantly changed in either CBHI (EGI+) or CBHII (EGII+) -overproducers. However, lack of EGI in

the CBHI-overproducer ALKO3761 or of EGII in the CBHII-overproducer ALKO3873 decreased the FPU activity by 10–15 %.

Production of CBHII by the CBH-overproducers ALKO4095 and ALKO4097 was increased by 3.9 and 3.4 fold, respectively, and thus approximately similar amounts of CBHII protein were produced using the *cbh1* or *cbh2* promoters (Table 3/III, Table 10). In ALKO4095, in which the *cbh1* promoter was used for *cbh2* expression, there was a slight decrease of CBHI.

3.2 Use of *T. reesei* preparations enriched with EGs and CBHs in cotton finishing (I, II)

3.2.1 Use of purified *T. reesei* cellulases in biostoning

The impact of the major *T. reesei* cellulases in biostoning was evaluated by Heikinheimo *et al.* (2000). According to their results, purified EGII was the most effective cellulase among EGI, EGII and CBHI at removing colour from denim. However, clearly higher enzyme dosage of the monocomponent EGII as compared to the whole cellulase preparation was needed to obtain an equal stone-washed effect. In this work the stone-washing effects of the cellulase preparation derived from the *T. reesei* strain VTT D-79125 (Table 8) with the addition of purified *Trichoderma* CBHI, CBHII, EGI and EGII were studied. VTT D-79125 produces all the identified *Trichoderma* cellulases, including the main cellulases CBHI, CBHII, EGI and EGII. These results have been published previously in US Patent 5,874,293.

The results showed that addition of EGII cellulase to the VTT D-79125 preparation increased the lightness, blueness and deltaE of denim fabric most clearly compared to the addition of other purified cellulases (Table 11). High deltaE and lightness indicate good biowashing performance. An increase in L value of about 1 unit can clearly be seen visually. Addition of CBHI or EGI improved the stone-washing effect as compared to the VTT D-79125 preparation but the effect was clearly lower than that obtained with EGII. In order to obtain the same stone-washed effect a double amount of CBHI or EGI was needed as compared to EGII. Addition of CBHII did not improve the stonewashing properties of the pure VTT D-79125 preparation.

Table 11. Colour measurements of the right side of denim fabrics treated with VTT D-79125 cellulase preparation and VTT D-79125 preparation fortified with purified cellulases.

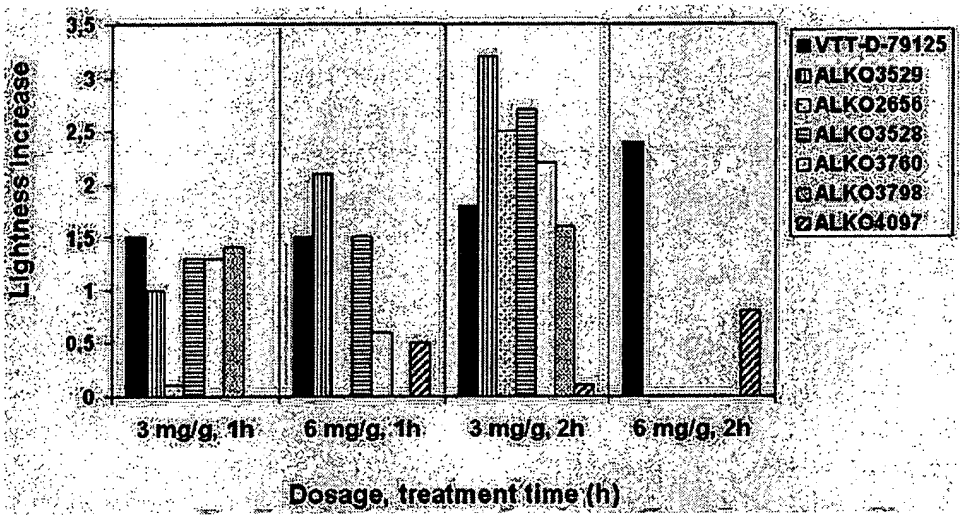
Cellulase added to VTT D-79125 preparation	Dosage mg / g	L	b	deltaE
1 hour				
-*	-	0.8	1.5	1.5
CBHI	1	0.8	2.0	1.5
CBHI	2	1.8	2.0	2.2
CBHII	1	1.1	1.7	1.6
CBHII	2	0.6	1.7	1.3
EGI	1	1.1	2.0	1.9
EGI	2	1.8	2.5	3.0
EGII	1	1.9	3.2	3.2
EGII	2	2.7	2.5	3.8
2 hours				
-*	-	1.2	1.9	1.5
CBHI	1	2.2	2.5	3.1
CBHI	2	1.4	2.5	2.7
CBHII	1	1.0	2.2	1.5
CBHII	2	1.3	2.7	2.6
EGI	1	2.2	2.9	2.2
EGI	2	2.0	3.1	3.5
EGII	1	2.5	3.7	1.9
EGII	2	4.2	3.5	5.2

*Pure VTT D-79125 preparation. The effects of the buffer on colours was deleted from these values. L: lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment. b: blueness unit of the fabric after treatment minus blueness unit of the fabric before the treatment. deltaE: colour difference in the L*a*b* colour space between the specimen colour and the target colour (target = untreated denim fabric).

3.2.2 Use of *T. reesei* preparations enriched with EGs and CBHs in biostoning (I)

Cellulase preparations derived from the EGII-overproducing strain ALKO3529, the EG-overproducing strain ALKO3528, the CBHI-overproducing strain ALKO3760, the CBHII-overproducing strain ALKO3798 and the CBH-overproducing strain ALKO4097 (Table 8) were compared in biostoning of denim fabric. The strain VTT D-79125 and the EGI-overproducing strain

ALKO2656 (containing three copies of the *egl1* expression cassette, Karhunen *et al.*, 1993) were used as controls. Results of the colour measurements of fabrics are shown in Figure 5. The results with EGII- and EG-overproducers have been presented in article I (Table 4) and with CBHI-, CBHII- and CBH-overproducers in US Patent 5,874,293.



The results are means of two duplicate treatments. The effects of the buffer on colours were deleted from these values. L: lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment.

Figure 5. Colour measurements of the right side of denim fabrics treated with VTT D-79125, ALKO3529, ALKO2656, ALKO3528, ALKO3760, ALKO3798 and ALKO4097 cellulase preparations.

The results showed that after 1 hour of treatment with the lower dosage almost the same stone-washed effects were obtained with ALKO3528, ALKO3529, ALKO3760 and ALKO3798 as with the strain VTT D-79125. When the dosage was increased to 6 mg/g (treatment time 1 h) or the time to 2 hours, the best effect was achieved with ALKO3529. After the 2 h treatment a slightly better stone-washing effect was also obtained with ALKO2656 and ALKO3528 as compared to the natural enzyme composition (VTT D-79125). The washing effects obtained with ALKO3760 or ALKO3798 were equal to that obtained with VTT D-79125. No clear increase in lightness units was obtained with the CBH-overproducing strain ALKO4097, in which the main endoglucanases had

been removed. Thus, a considerably lower dosage of cellulases from endoglucanase-overproducing strains, especially from the EGII-overproducing strain, was needed to achieve a comparable stone-washing effect as with the cellulases of VTT D-79125.

3.2.3 Use of *T. reesei* preparations enriched with EGs and CBHs in biofinishing (II)

Cellulase preparations derived from the EGII-overproducer ALKO3529, the EG-overproducer ALKO3528, the CBHI-overproducer ALKO3760, the CBHII-overproducer ALKO3798 and the CBH-overproducer ALKO4097 (Table 8) were used in biofinishing of cotton fabric. Cellulase from the strain VTT D-79125 was used as a control. The effects of the different cellulases were evaluated by measuring the weight loss and analyzing the pilling performance and visual appearance (amount of fuzz and pills on the fabric surface) of the fabric. The results showed (Table 4/II) that the best visual appearance and the greatest reduction in pilling tendency was achieved with the EGII-overproducing ALKO3529 preparation when comparing the same dosages of different preparations. Moreover a considerably smaller dosage of this preparation was needed as compared to the other preparations. Furthermore, by using the CBHII-overproducing strain ALKO3798 the visual appearance of the cotton fabric could be improved and the pilling tendency could be reduced as compared to the effects achieved with VTT D-79125 or the CBHI-overproducing strain ALKO3760. Almost equal effects were obtained with ALKO3798 and the EG-overproducing strain ALKO3528.

3.3 Novel neutral cellulases for biostoning (III)

The use of *T. reesei* acid cellulases in biostoning has been limited due to the high backstaining effect and weakening of the fabric. Neutral cellulases available on the market have been less aggressive and have not been affected by the pH increase during denim finishing, but are reported to require a longer wash time than *Trichoderma* cellulases (Klahorst *et al.*, 1994). In this part of the work novel cellulases were screened for the denim finishing application. New cellulases with the following characteristics are still needed:

• active over a broad pH range leading to less need for a buffering system to control pH,
• short reaction time,
• no significant weakening of the fabric,
• good finishing properties, e.g. low backstaining.

When acid cellulases are used, an efficient buffering system is always needed because alkalinity increases during denim washing as the caustic soda used in dyeing is released from the fabric. Incoming water in wet processing is also typically in the neutral range (Solovjeva *et al.*, 1998).

A laboratory scale biostoning assay was used for screening of microorganisms and in conjunction with the purification of cellulases. High lightness increase on the right side of the fabric was used as an indication of good indigo-dye release and stonewashing effect. Backstaining was quantified by measuring the colour values on the reverse side of the fabric, i.e. low lightness and high blueness values indicated high backstaining.

3.3.1 Screening of microorganisms producing neutral cellulases

Various fungal species were screened for identification of extracellular cellulases acting over a broad pH range (pH 5–8, especially at pH 7). The culture supernatants from about 25 fungal strains (Budapest University of Technology and Economics, Hungary / III) were tested for endoglucanase activity at neutral pH (pH 7.0, data not shown). Preparations derived from the thermophilic fungal strains *M. thermophila* ALKO4179, *M. albomyces* ALKO4237, *C. thermophilum* ALKO4265 and *S. thermophilum* ALKO4125 were chosen for biostoning experiments. *S. (Chrysosporium) thermophilum* and *M. thermophila* are the anamorph and teleomorph stages of the same fungus (Maheshwari *et al.*, 2000).

In the biostoning assay in neutral conditions the lightness units were increased on the right side of the denim fabric washed with culture supernatants of *M. albomyces, M. thermophila* and *S. thermophilum,* showing effective release of indigo-dye (Table 3/III, summary in Table 12). Denim fabric treated with the culture supernatant of *C. thermophilum* did not lighten, but the blueness unit was increased. This is probably an indication of cellulase action on denim, but

resulting at the same time in high backstaining. A commercial acid cellulase product of *T. reesei* (Ecostone L, Primalco Ltd. Biotec) did not produce a stonewashing effect at pH 7. Practically no backstaining was measured from the fabrics treated with supernatants of *M. albomyces, M. thermophila* and *S. thermophilum* as compared to *C. thermophilum* or Ecostone L (pH 5.2 and 7) (Table 3/III, Table 12). Because the best stone-washing effect with the lowest backstaining was obtained with the preparation derived from *M. albomyces*, this fungus was chosen for isolation of cellulases for stone-washing in neutral conditions.

According to information in the website www.indexfungorum.org/Names/NAMES.ASP, *M. albomyces* is a fungal ascomycete and its phylogenetic classification is: Incertae sedis, Sordariales, Sordariomycetidae, Ascomycetes. According to Guarro *et al.* (1996) *Melanocarpus* belongs to the non-ostiolate Sordariales and its closest relatives are the genera *Thielavia, Chaeromidium, Boothiella, Corynascus* and *Corynascella.*

Table 12. Colour measurements of denim fabric treated with different culture supernatants.

Source of enzyme	*Treatment pH*	*ECU/g of fabric*	L_{right}	$b_{reverse}$
-	7	-	2.1	-1.1
M. albomyces	7	200	5.5	2.3
M. thermophila	7	200	4.4	2.2
S. thermophilum	7	200	3.5	1.4
C. thermophilum	7	200	3.3	6.6
Ecostone L	5.2	200	2.0	4.8

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

3.3.2 Purification and properties of three cellulases of *M. albomyces*

Culture supernatant of *M. albomyces* ALKO4237 was fractionated on DEAE Sepharose and the protein pools were tested in biostoning. According to the results two endoglucanases with molecular masses of about 20 and 50 kDa and a third protein with a molecular mass of about 50 kDa, but no endoglucanase

activity, were responsible for the biostoning activity (data not shown). The three cellulases were purified to homogeneity (Tables 1 and 2/III). The 20 kDa endoglucanase crystallized spontaneously when the SP-Sepharose eluates were stored for a few days at 7°C. The washed crystals showed a single band on SDS-PAGE corresponding to a molecular mass of 20 kDa (Fig. 1/III). The 20 kDa endoglucanase was a relatively heat-stable cellulase and had a high pH optimum. At 50°C it exhibited 80 % or more of its maximum activity over the pH range 4–9 and between pH 5.5 and 7.5 the enzyme was more active at 70 °C than at 50 °C (Figure 3/III).

The purified 50 kDa endoglucanase (Figure 1/III) had activity both against hydroxyethyl cellulose and MUL (CBHI). This EG was also active over a broad pH range: at 50 °C a constant activity was observed between pH 4.4 and 7 and at 70 °C the optimum was at pH 6 (Figure 4/III). The third protein was the 50 kDa cellobiohydrolase with a low activity against MUL (CBHI) at pH 5–7 but no measurable endoglucanase activity.

Amino acid sequences of tryptic peptides derived from the 20 kDa EG and the 50 kDa CBH and from CNBr-digested 50 kDa EG (Table 4/III) showed homology towards e.g. *Humicola* and *Fusarium* cellulases.

Table 13. Colour measurements of denim fabric treated at pH 7 with purified Ma 20 and 50 kDa endoglucanases and 50 kDa cellobiohydrolase.

Sample	Dosage mg protein / g fabric	L_{right}	$b_{reverse}$
-	-	2.8	1.6
20 kDa EG	0.18	5.6	4.0
50 kDa EG	0.15	2.6	1.0
50 kDa CBH	0.15	2.7	0.5
20 kDa EG + 50 kDa EG	0.18 + 0.075	5.6	2.5
20 kDa EG + 50 kDa CBH	0.18 + 0.15	4.7	3.0

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

At a protein dosage of $1/80^{th}$ of that of unfractionated *M. albomyces* culture filtrate, the purified 20 kDa EG resulted in the same degree of lightening in biostoning at neutral pH (Table 5/III, summary in Table 13). Furthermore the purified 20 kDa protein caused less backstaining on the reverse side than the

unfractionated culture filtrate. The 50 kDa EG or 50 kDa CBH did not alone release indigo-dye (Table 6/III, Table 13). However, both enzymes decreased backstaining on the reverse side when used in combination with 20 kDa endoglucanase.

3.4 Cloning of neutral cellulase genes of *M. albomyces* and their expression in *T. reesei* (IV)

The genes coding for the three cellulases of *M. albomyces* ALKO4237 which were effective in biostoning at neutral pH (III) were subsequently cloned, sequenced and expressed in *T. reesei* for use of the cellulase preparations produced by the transformants in biostoning of denim.

3.4.1 Cloning and characterisation of the genes

Degenerated primers (Table 1/IV) based on the peptide sequences (Table 4/III) of the purified proteins were used to amplify the genes coding for the 20 kDa and 50 kDa EGs and the 50 kDa CBH of *M. albomyces*. The amplified fragments obtained encoded the majority (in the case of 20 kDa EG) or one (50 kDa EG, 50 kDa CBH) of the cellulase-derived peptides.

The genomic library of *M. albomyces* was screened with each of the amplified fragments and the positive clones were identified. The 849 bp open reading frame (ORF) of 20 kDa EG codes for 235 amino acids, is disrupted by two predicted introns and predicts a protein of 25 kDa for the full-length preprotein and 22.9 kDa for the mature protein. The 1364 bp ORF of 50 kDa EG codes for 428 amino acids, has one intron and predicts a molecular weight of 46.8 kDa for the full-length protein and 44.8 kDa for the mature protein. The 1735 bp ORF of 50 kDa CBH codes for 452 amino acids, has five introns and predicts a molecular weight of 49.9 kDa for the full-length protein and 47.6 kDa for the mature protein. All the peptides sequenced from each of the purified cellulases (III) were found in the corresponding predicted protein sequences. The cloned cellulases lack a consensus cellulose binding domain and its associated linker.

The 20 kDa EG belongs to the family 45 of GH (Henrissat *et al.*, 1998) and was named *Ma* Cel45A-cellulase (EGV). The 50 kDa EG and 50 kDa CBH belong to the family 7 of GH and were named *Ma* Cel7A (EGI) and *Ma* Cel7B (CBHI), respectively. *Ma* Cel45A-cellulase is similar to *Thielavia terrestris* endoglucanase (76% identity in a 234 amino acid overlap) and *H. insolens* endoglucanase V (76 % identity in a 235 amino acid overlap) (Table 2/IV, Fig. 6). The proposed active site aspartates are at positions 10 and 120 of the mature protein (Fig. 6). The *Ma* Cel7A protein is similar to *Humicola grisea* endoglucanase I (about 73 % identity in a 416 aa overlap) and the *Ma* Cel7B to *H. grisea* cellobiohydrolase (78 % identity in a 449 aa overlap) (Table 2/IV).

3.4.2 Heterologous production in *T. reesei*

Plasmids pALK1231 and pALK1235 were constructed for expression of the *Ma cel45A* (*egl5*) gene in the *cbh1* and *egl1* loci of *T. reesei*, respectively, plasmids pALK1238 and pALK1240 for expression of the *Ma cel7A* (*egl1*) gene in the *cbh1* and *egl1* loci and plasmid pALK1242 for expression of the *Ma cel7B* (*cbh1*) gene in the *cbh1* locus (Fig. 1/IV, Table 7). The genes were expressed from the *cbh1* promoter of *T. reesei* individually in an EGII-negative *T. reesei* host ALKO3620. *T. reesei* EGII has been shown to weaken the strength properties of cotton fabrics significantly more than CBHI or EGI (Miettinen-Oinonen *et al.*, 1996, Heikinheimo *et al.*, 1998). Because of this *T. reesei* deficient in EGII was used as a host in transformations. The replacement frequencies, detected from the transformants by dot blotting and immunostaining, of the *cbh1* locus varied between 24 and 80 % and of the *egl1* locus between 15 and 23 % depending on the plasmid. Seven transformants were studied in more detail.

The genome analysis (Southern hybridization) of Cel45A transformants showed that in the CBHI-negative transformants ALKO3620/1231/14 and ALKO3620/1231/16 the *cbh1* gene of *T. reesei* was replaced by one copy of the expression cassette – *amd*S marker fragment of pALK1231 and in the EGI-negative transformants ALKO3620/1235/40 and ALKO3620/1235/49 the *egl1* gene was replaced by one copy of the expression cassette – *hygB* marker fragment of pALK1235. Thus, the first two strains are unable to produce *T. reesei* CBHI and EGII. The latter two strains are unable to produce EGI and EGII.

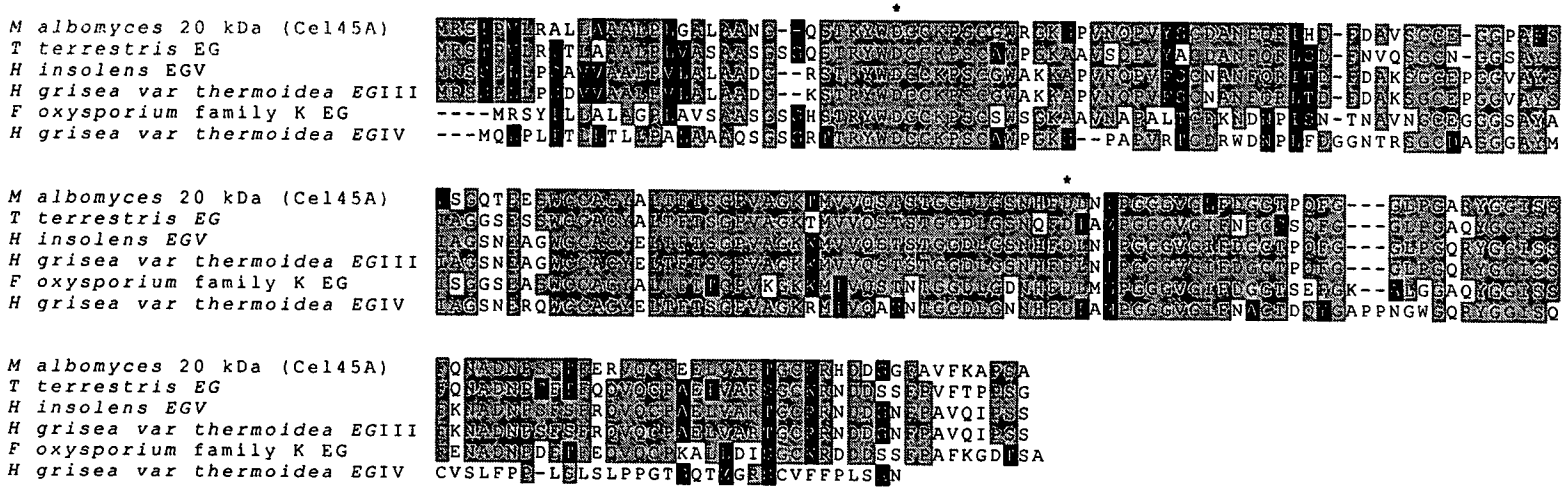


Figure 6. Alignment of the M. albomyces Cel45A with homologous endoglucanases. Identical residues are depicted in grey and similar residues in a black background. The complete genus names are shown in Table 2/IV. Only the catalytic domains are shown. The catalytic active site aspartates are indicated with stars. Previously unpublished.

Cel45A (EGV) transformants were cultivated on cellulase-inducing medium. The pH at the end of the cultivation was about 3. In these conditions Cel45A could be detected only by Western blotting with Cel45A-specific polyclonal antibody and in samples extracted from the mycelium. When the pH of the cultivation medium was maintained above 4 by using 5% KH_2PO_4, Cel45A could be detected from the culture medium by Western blotting and the size was the same as the size of the purified protein (Fig. 2/IV). Single copy replacement transformants of Cel45A produced several times higher endoglucanase activity levels as compared to the parent strain *M. albomyces* ALKO4237 (Table 3/IV). The Cel45A-transformants produced 2100–2500 NCU/ml (activity against carboxyethyl cellulose, pH 7) at 70°C. The integration site of the expression cassette had no significant effect on the activity levels.

Proteolytic degradation of the secreted *Ma* Cel45A in the *T. reesei* culture at pH < 4 was tested by mixing purified Cel45A into the culture supernatant of *T. reesei* ALKO3620 (pH about 3) and incubating at 30 °C. The Cel45A band intensity detected by Western blotting decreased with time and disappeared after 60 min (Fig. 3/IV). When an aspartic protease inhibitor Pepstatin A alone or together with a serine and cysteine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added into the culture supernatant of *T. reesei,* no degradation of Cel45A was observed (Fig. 3/IV). When PMSF was added into the culture supernatant alone, degradation of Cel45A still occurred. Thus the probable cause of *Ma* Cel45 degradation was an aspartic protease.

One copy of the pALK1238 expression fragment containing the *Ma cel7A* (*egl1*) gene had replaced the *cbh1* gene in *T. reesei* transformant ALKO3620/1238/42 and one copy of the pALK1240 expression fragment containing *cel7A* had replaced the *egl1* gene in ALKO3620/1240/32. The activity against CMC at pH 7, 70° produced by ALKO3620/1238/42 in cellulase-inducing conditions (pH 4) was higher (6000 NCU/ml) than that produced by ALKO3620/1240/32 (3500 NCU/ml) (Table 3/IV). Thus, a higher production level was obtained when the expression cassette had integrated into the *cbh1* locus compared to the *egl1* locus. ALKO3620/1240/32 produces Cel7A (EGI) protein with the same size as the purified native protein (Fig. 2/IV).

In transformant ALKO3620/1242/13 the expression fragment of pALK1242 containing the *Ma cel7B* (*cbh1*) gene had replaced *cbh1* of *T. reesei.* The

transformant produced slightly higher activity against MUL (CBHI) than the control strain ALKO4072 in which the *cbh1* and *egl2* genes had been deleted (Table 3/IV). The transformant produced *Ma* Cel7B (CBHI) protein with the same size as the purified native protein (Fig. 2/IV).

3.4.3 Use of the heterologous *Ma* Cel45A in biostoning

The purified *Ma* Cel45A (EGV) alone was able to effectively impart a stonewashed appearance to denim fabric (Tables 5–6/III, Table 13). Therefore endoglucanase preparations produced by the Cel45A transformants were tested in biostoning in neutral conditions. Preparations produced by the transformants ALKO3620/1231/14 and ALKO3620/1231/16 combined (CBHI-, EGII-) and ALKO3620/1235/49 (EGI-, EGII-) increased the release of indigo dye from the right side of the denim fabric, resulting in a lightening of 6.1 (L_{right}) with 400 ECU/g dosage after two hours treatment (Table 4/IV, summary in Table 14). At pH 7 the stone-washing effect of heterologous *Ma* Cel45A preparations was significantly better and the backstaining was clearly lower compared to the effects obtained with the acid cellulase product Ecostone L at pH 5.

Table 14. Colour measurements of denim fabric treated with T. reesei cel45A-transformants and Ecostone L (Primalco Ltd. Biotec).

Preparation	*Treatment pH*	*ECU/g of fabric*	L_{right}	$b_{reverse}$
Buffer only	7	-	2.6	0.2
ALKO3620/1235/49	7	100	4.7	2.5
	7	400	6.1	3.6
ALKO3620/1231/14+16	7	100	4.4	1.5
	7	400	6.1	2.4
Ecostone L	5.2	200	2.0	4.8

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

4. Discussion

4.1 Homologous production of cellulases by *T. reesei*

The biotechnically important filamentous fungus *T. reesei* was exploited as a host in efficient production of homologous cellulases. Different tailored strains were constructed in order to obtain specific industrial cellulase preparations for commercial applications in biostoning and biofinishing. In total, eight strains producing homologous *T. reesei* cellulases with different profiles were constructed.

Production of the native natural endoglucanases and cellobiohydrolases of *T. reesei* was improved individually by using the *Trichoderma cbh1* promoter and by adding extra copy numbers of the *egl2*, *cbh1* or *cbh2* genes. As a result high EGII, CBHI or CBHII activity-producing strains with variable backgrounds of other cellulases were obtained. In shake flask cultivations in inducing conditions the best EGII-overproducing strain ALKO3529 produced EGII protein 3.2 times more than the parent strain, whereas the best CBHI-overproducing strain ALKO3760 produced 1.5-fold more CBHI and the best CBHII-overproducing strain 3.9-fold more CBHII than the parent strain (Table 15). Previously, the highest reported overproduction level of homologous cellulase in *T. reesei* has been 2.2 g EGI/l, which is three times higher than in the parent (Karhunen *et al.*, 1993).

Table 15. Amounts of cellulases overproduced by the best T. reesei strains overproducing homologous cellulases individually under the cbh1 promoter.

Strain	Cellulase composition*	Amount
ALKO3529	CBHI, CBHII, EGI, **EGII**$^{+}$	1.3 g EGII / l, 3.2x parent
ALKO3760	**CBHI**$^{+}$, CBHII, EGI, EGII	3.4 g CBHI / l, 1.5x parent
ALKO3798	CBHI, **CBHII**$^{+}$, EGI, EGII	0.7 g CBHII / l, 3.9x parent

*Overproduction is indicated as +, of the main cellulases

Factors reported to affect overproduction of proteins in filamentous fungi include promoter efficiency, copy number of the expression cassette and integration site (Verdoes *et al.*, 1995, Archer and Peberdy, 1997, Nevalainen *et al.*, 2004). In this work the *cbh1* promoter was used for homologous cellulase

expression in *T. reesei.* The site of integration was not observed to have a significant effect on the CBHII production levels of the CBHII-overproducer transformants or on the EGII production levels of the EGII-overproducer strains. However, copy number of the expression cassette did have an effect on the production levels, depending on the expressed gene. In the EGII-overproducing strains the increase in the production of EGII protein and endoglucanase activity followed the copy number of the *egl2* expression cassette (total up to three *cbh1* promoters). Simultaneous inactivation of *cbh1* did not increase significantly the EGII production or the endoglucanase activity levels in the studied EGII-overproducing strains. In the case of CBHI-overproduction some limiting factors appeared already with one additional copy of the *cbh1* expression cassette (a total of two *cbh1* promoters), since one additional copy of the *cbh1* expression cassette increased the production of CBHI protein and MUL (CBHI) activity only about 1.3 fold and two copies about 1.5 fold compared to the parent. In the strains used as hosts in this work, CBHI accounted for 70–80% of the secreted cellulases (Table 2/I, Table 1/II). Thus, although CBHI is the major secreted protein of *T. reesei* its production could be further increased, but the increase was not linear according to the copy number of the expression cassette. It is also notable that there was an increase in the amount of total secreted protein in the CBHI-overproducer strain, indicating that *T. reesei* has a capacity for increased overall protein production levels. Interestingly, in the CBHII-overproducing strains one copy of CBHII under the *cbh1* promoter increased production of CBHII more than twofold (Table 15). Karhunen *et al.* (1993) suggested that the third copy of the *cbh1* promoter would be enough to titrate out regulatory proteins or other essential transcription factors in overproduction of EGI. In *Aspergillus niger,* copy number-dependent improvement in the levels of secreted glucoamylase has been observed with up to about 20 copies (Archer and Peberdy, 1997).

The overproduction of different endogeneous cellulases (other cellulases present) had effects on the cellulase activities and on the production levels of the other main cellulases. FPU activity is mainly produced by cellobiohydrolases and is also an indication of total hydrolysis of cellulose. EGII was found to affect the FPU activity by increasing it about 10% in the EGII-overproducer (*cbh1* gene present, ALKO3529) compared to the parent strain. Increase in FPU activity was also observed in fermenter culture supernatant of the CBHII-

overproducing transformant ALKO3799 (data not shown). No increase in FPU activity was observed in CBHI-overproducing strains.

As expected the deletion of *egl1* or *egl2* genes decreased (50 %) the endoglucanase activity in CBHI- or CBHII-overproducing transformants. However, endoglucanase activity was also decreased (20–30 %) in the EGII-positive CBHII-overproducers and the EGI-positive CBHI-overproducers. The amounts of EGI and CBHII were decreased in EGI-positive CBHI-overproducing transformants. In CBHII-overproducing transformants the amount of secreted CBHI was clearly decreased (both EGII+ and - background). In one EGII-overproducing strain (CBHI-positive) a very slight reduction in the amount of secreted CBHI was observed. These might be indications of mutual cellulase regulation or possibly of a secretion stress. Saloheimo *et al.* (2003b) recently showed that in *T. reesei* the genes encoding secreted proteins are rapidly downregulated concurrently with induction of the UPR pathway. However, our data suggests that overproduction of individual *T. reesei* cellulases can be performed successfully without the possible interference of UPR. As an example EGII was overproduced according to the copy number of the expression cassette and no significant reduction in the amounts of other main cellulases was detected. However, UPR might have some role, because the amount of proteins overproduced was not always linear, e.g. when overproducing CBHI, and the amounts of the other main cellulases may decrease as described above.

One additional copy of the *egl2* expression cassette in the *cbh*1 locus increased the endoglucanase activity by 2.3 fold compared to the parent strain, whereas one additional copy of the *egl1* gene expressed under the *cbh1* promoter in the *cbh*1 locus increased the endoglucanase activity by 1.9 fold as shown by Karhunen *et al.* (1993). By further increasing the copy number of *egl2* a greater increase in endoglucanase activity was obtained as compared with the corresponding copy number of the *egl1* expression cassette. Thus it can be concluded that EGII has a major impact on the endoglucanase activity measured as activity against HEC. This is consistent with the higher specific activity of EGII than of EGI on HEC and β-glucan (Suurnäkki *et al.*, 2000) and with the results obtained with cellulase deletion strains of *T. reesei* (Suominen *et al.*, 1993).

In addition to the strains overproducing EGII, CBHI and CBHII separately, strains overproducing the main endoglucanases EGI and EGII without

cellobiohydrolases and strains overproducing CBHI and CBHII without the main EGs were constructed. Endoglucanases without any cellobiohydrolases can be produced with the strain ALKO3528 which produces, as expected, endoglucanase activity fourfold higher than the parent strain. In culture supernatant of the CBH-overproducers some endoglucanase activity was detected due to the minor EGs still present (Table 3/II). In the CBH-overproducing transformants the host strain ALKO3761 contained two copies of the *cbh1* promoter and when *cbh2* was expressed in this background with either *cbh1* or *cbh2* promoters similar increases in CBHII were obtained. However, the simultaneous use of the *cbh1* promoter for both *cbh1* and *cbh2* expression in one strain decreased the amount of secreted CBHI as compared to the use of the *cbh2* promoter, suggesting limitations in the *cbh1* promoter.

4.2 Novel neutral cellulases

There is a continuing need for new cellulases with a variety of characteristics for use in different industrial applications and conditions such as in treating textiles. Cellulases acting over broad pH ranges with near-neutral optima and causing low backstaining would be preferable in biostoning. In the present work, new cellulases for finishing of cotton-containing denim articles were screened, isolated and characterized and the genes encoding them were cloned and sequenced.

Enzymatic washing results of denim cannot be predicted simply by measuring the cellulase activities in the preparations (Gusakov *et al.*, 2000b). In this work the laboratory-scale biostoning method was successfully used in screening and purification of novel neutral cellulases for finishing of denim fabric. When preparations produced by the strains *Melanocarpus albomyces, Myceliophthora thermophila, Chaetomium thermophilum* and *Sporotrichum thermophilum* were compared, the preparation of *M. albomyces* was found to be the most effective in releasing indigo dye from denim and to cause lowest backstaining. Backstaining was earlier claimed to depend on pH, with lower redeposition at pH 7 than at pH 5 (Tyndall, 1990), but further studies have indicated that the nature of the enzyme has an impact on backstaining (Klahorst *et al.*, 1994, Gusakov *et al.*, 1998). Our studies clearly showed differences in performance and especially in backstaining between the strains (Table 12). The culture supernatant of *C.*

thermophilum caused extremely high backstaining at pH 7, contrary to the culture supernatants of *M. albomyces, M. thermophila* or *S. thermophilum,* confirming the hypothesis that backstaining is dependent on the origin of the cellulase preparation and the functional properties of cellulases rather than on the pH of the treatment solution.

M. albomyces produces at least three cellulases with an effect on the biostoning process, and all three were purified. The purified enzymes were 20 kDa endoglucanase (Cel45A / EGV), 50 kDa endoglucanase (Cel7A / EGI) and 50 kDa cellobiohydrolase (Cel7B / CBHI). Low hydrolysis of MUL was the only enzyme activity found for Cel7B. As this protein had sequence similarities to *H. grisea* cellobiohydrolase I it was named cellobiohydrolase. The isolated Cel45A and Cel7A endoglucanases are stable enzymes that exhibit endoglucanase activity over a wide range of pH values and at high temperatures. Especially Cel45A is a relatively heat stable enzyme. These properties increase the application potential of the endoglucanases in many conditions and applications, such as in household detergents in which cellulases should act at higher pH values (pH 7–12) and temperatures (40–90 °C).

The genes encoding the three isolated cellulases of *M. albomyces* were cloned and sequenced. The protein sequences deduced from the gene sequences showed significant sequence similarity with other fungal cellulases (Table 2/IV, Fig. 6), but lacked a consensus CBM and a linker region. *Ma* Cel45A (EGV) is similar to *T. terrestris* endoglucanase and *H. insolens* EGV (Cel45A). *Ma* Cel7A (EGI) is similar to *H. grisea* EGI and *Ma* Cel7B (CBHI) to *H. grisea* CBH.

According to Schülein *et al.* (1996), when *T. terrestris* EG was used in denim finishing (pH 5) an almost bleached appearance could be obtained. In addition to cellulases of *T. terrestris* and *T. reesei,* cellulases from various fungal sources have been reported to be applicable to denim finishing at different pH values (Table 6). Different finishing effects have been achieved depending on the enzyme. The cellulases (Table 6) for which the sequence is available and the cellulases of *M. albomyces* were compared in the predicted phylogenetic tree (Fig. 7). In addition to *Ma* Cel45, high abrasion of denim was reported to be achieved with EGV of *H. insolens* (Schülein *et al.,* 1998, pH 7), EG of *T. terrestris* (pH 5) and *Acremonium* sp. (Schülein *et al.,* 1996, pH 7) and with *M. thermophila* EG attached to *H. insolens* EGV CBM and linker (Schülein *et al.,*

1996, pH 6). These cellulases appear to be clustered closer to each other than to the cellulases with reported effects of little (*H. insolens* EGI and *F. oxysporium* EGI, Schülein *et al.*, 1998) or no (*M. albomyces* Cel7A and Cel7B, this work, *T. reesei* CBHI, Heikinheimo *et al.*, 2000) abrasion. Interestingly, although high abrasion is obtained at pH 5 with *T. reesei* EGII (Heikinheimo *et al.*, 2000, this work), it seems to be very distant from *M. albomyces* Cel45A working efficiently at pH 7 and also at pH 5 (data not shown). However, the backstaining levels are significantly higher with *T. reesei* cellulases as compared to *Ma* Cel45A (Table 4/IV, Table 14). *H. insolens* EGI and *F. oxysporium* EGI have been claimed to prevent backstaining in denim wash (Schülein *et al.*, 1998; no results were presented). This was shown in the case of *Ma* Cel7A. *Ma* Cel7A prevents backstaining when used together with *Ma* Cel45A (Table 13). *Ma* Cel7A, *H. insolens* EGI and *F. oxysporium* EGI appear to be situated close to each other in the predicted phylogenetic tree (Fig. 7).

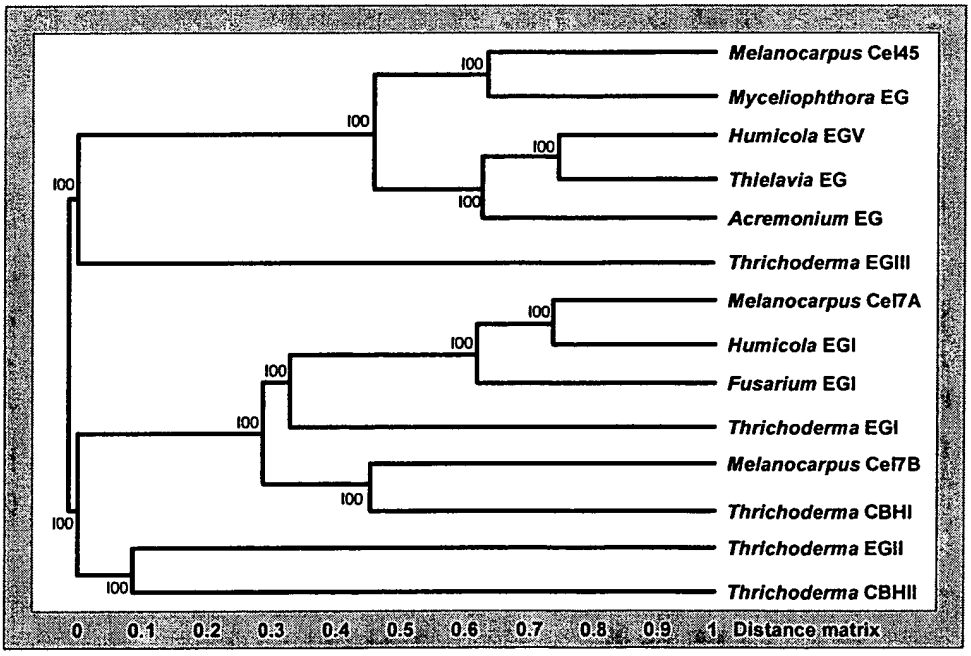


Figure 7. The predicted phylogenetic tree (with bootstrap values) of cellulases tested in biostoning. The tree was made with the GeneBee – Molecular Biology Server with the TreeTop – Phylogenetic Tree prediction program. Cluster algorithm.

In this work a rather labour-intensive screening method was used to look for novel cellulases for biostoning. If more novel cellulases with similar performance are desired, these could be searched by looking for genes which are homologous with the genes encoding the cellulases of *M. albomyces* (especially Cel45A) or closely related genes based on phylogenetic analysis. The cellulases thus identified could then be tested in the application.

4.3 Heterologous production of cellulases by *T. reesei*

The cellulase production capacity of *M. albomyces* is far too low for efficient and cost-effective industrial-scale production. Therefore *T. reesei* was chosen as a potential host for production of Cel45A (EGV), Cel7A (EGI) and Cel7B (CBHI) of *M. albomyces*. In this work five strains producing heterologous *M. albomyces* cellulases with different profiles were constructed. *M. albomyces* cellulases were produced specifically for biostoning at neutral pH. *Trichoderma*'s own cellulases can have undesired effects (backstaining, strength loss) in biostoning (Klahorst *et al.*, 1994, Heikinheimo and Buchert, 2001), especially if the pH of the washing liquor is decreased below 6–7. Cellulases were expressed under the *T. reesei cbh1* promoter in *T. reesei* hosts lacking EGII and either EGI or CBHI and single copy transformants produced commercially significant levels of *M. albomyces* cellulases even in shake flask cultures: approximately 0.3–0.8 g Cel45A/l and 1–1.5 g Cel7A/l were produced. The proteolytic degradation, caused by aspartic protease, of recombinant *Ma* Cel45A had to be prevented by maintaining the pH of the cultivation medium above 4. Proteolytic degradation has been one reason for low yields of heterologous products in filamentous fungi (Nevalainen, 2001). The characteristics of the recombinant *Ma* Cel45A preparations were suitable for biostoning in neutral conditions. Published secreted yields of fungal enzymes heterologously expressed in *T. reesei* from the *cbh1* promoter have varied from no detectable protein to a few grams depending on the source of the gene (Paloheimo *et al.*, 1993). For example expression of *Aspergillus niger* acid phosphatase yielded 0.5 g/l in shake flasks (Miettinen-Oinonen *et al.*, 1997), *Hormoconis resinae* glucoamylase 0.7 g/l (Joutsjoki *et al.*, 1993) and *Phlebia radiata* laccase about 3 mg/l (Saloheimo and Niku-Paavola, 1991). A few published reports exist on the expression of genes from thermophilic fungi or bacteria in *T. reesei* using the *cbh1* promoter (reviewed in Penttilä *et al.*, 2004). The *xyn2* gene encoding

xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea* was fused to the CBHI secretion signal and the XYNII enzyme was shown to be correctly processed in the *T. reesei* host (de Faria *et al.*, 2002). The highest level of XYNII production was about 0.5 g/l, approximately similar to the production of *Ma* Cel45A in *T. reesei* under the *cbh1* promoter (no fusion). Expression of *M. albomyces* laccase in *T. reesei* under the *cbh1* promoter resulted in 0.2 g laccase/l (Kiiskinen *et al.*, 2004). Thus, the amount of *Ma* Cel7A produced by *T. reesei* (1–1.5 g/l) is the highest level of heterologously expressed fungal protein hitherto reported. Production economics of *T. reesei* strains producing *M. albomyces* cellulases may be further improved by adding more copies of the respective gene, by using other mutant strains as hosts, by using complex industrial media and appropriate fermenter cultivation strategies and by random mutagenesis and selection of the production strains.

4.4 Cotton finishing

One of the most important objectives of biostoning and biofinishing processes is to carry out the treatment processes cost-effectively. Furthermore, it is essential to achieve a good appearance of the textile while preserving the strength properties. Cellulases derived from *T. reesei* are commonly employed in textile treatments and investigations have been made of the effects of different cellulase ratios in the treatments (Clarkson *et al.*, 1992a, b, c, 1993, 1994b, Kumar *et al.*, 1997, Heikinheimo *et al.*, 1998, Heikinheimo and Buchert, 2001, Table 6).

In this work the role of the main cellulases of *T. reesei* in textile applications was further elucidated in order to identify suitable cellulase combinations for industrial biofinishing and biostoning. Each of the purified main cellulases was added to the normal overall cellulase composition of *T. reesei*. When the cellulase composition with an elevated content of EGII was used for treating cotton fabric and denim fabric, improved biofinishing effect and stone-washed appearance were obtained as compared to the total cellulase composition or compositions containing elevated amounts of EGI, CBHI or CBHII. These results are consistent with those studies in which purified EGII was found to be the most effective of the *T. reesei* main cellulases in biostoning and biofinishing (Heikinheimo *et al.*, 1998, Heikinheimo *et al.*, 2000, Heikinheimo and Buchert, 2001). The phenomenon was confirmed by comparing cellulase preparations

derived from *T. reesei* strains producing enhanced EGI, EGII, both EGI and EGII, CBHI, CBHII or both CBHI and CBHII in biostoning and biofinishing (I, II). Cellulase preparation derived from the EGII-overproducing strain ALKO3529 improved the stone-washing effect and biofinishing performance compared to its parent strain VTT D-79125 or to the other overproducing strains, when the same enzyme (protein) dosage and time were used (Table 4/II, Fig. 5). Furthermore, the same stone-washing effect could be obtained, with considerably lower enzyme dosage and time, when using the EGII-enriched preparation than when using the preparation produced by the parental strain. Thus, it is possible to achieve improved stone-washing and biofinishing effects by utilizing the production strain producing an increased relative amount of EGII in the total cellulase mixture. Preparations produced by the EGII-overproducer strains described in this work have also been used together with different experimental *T. reesei* cellulase preparations for biofinishing of different types of fabrics (Miettinen-Oinonen *et al.*, 2001). EGII-based preparations (background CBHI+/-) gave the highest pilling removal. The improvement was not dependent on the ratios of CBH and EG, but an increased level of EGII appeared to be responsible for the improvement.

Use of the EGII-enriched preparation in denim finishing and biofinishing allows shorter processing times, i.e. the EGII-preparation acts more rapidly than the other tested preparations. This would mean more time- and cost-effective treatment procedures and savings in equipment as well as treatment facilities. Another advantage may be reduction of the amount of enzyme required in the treatment solution leading to savings in enzyme costs.

Endoglucanases are known to be key enzymes in biostoning and biofinishing applications (Kumar *et al.*, 1997, Heikinheimo *et al.*, 1998, Heikinheimo *et al.*, 2000, Liu *et al.*, 2000), but strength and weight losses have been negative impacts often associated with endoglucanase treatments (Heikinheimo and Buchert, 2001). Attempts to minimize the strength losses caused by *T. reesei* cellulases have been made by altering the ratios of EG and CBH in the cellulase preparation (Clarkson *et al.* 1992a, b, 1993). Although a cellulase preparation with increased levels of especially EGII is the most promising in biostoning and biofinishing applications, improved performance in biofinishing was also observed with the preparation from the CBHII-overproducing strain as compared to the wild type (Table IV/II). However, no difference in weight loss of the

fabric was observed when EGII- and CBHII-enriched preparations were compared at the same depilling level and dosage. The improved performance attained with the CBHII-preparation might be explained by the synergistic action of cellulases (Heikinheimo and Buchert, 2001) or possibly by the low β-glucanase activity of CBHII (Henriksson *et al.*, 1995).

T. reesei cellulases are useful in denim finishing to impart a certain type of stonewashed appearance, but they have disadvantages such as a tendency to promote backstaining and weakening of fabrics. Cellulases of *M. albomyces* were shown to have a good capability to impart stonewashed appearance in denim. Of the isolated *M. albomyces* cellulases Cel45A (EGV) was mainly responsible for the good stone-washing effect of *M. albomyces* ALKO4237 growth medium, with reasonably low backstaining. *Ma* Cel45A is also an excellent cellulase for biostoning applications because of its broad operational pH and temperature ranges. The superiority of *Ma* cellulases over *T. reesei* cellulases with regard to backstaining was clearly demonstrated (Tables 3/III, 4/IV, 12, 14). Cellulase preparation containing heterologous *Ma* Cel45A performed well in biostoning at neutral pH and an equal degree of abrasion and equally low backstaining were obtained as with the purified *Ma* Cel45A.

M. albomyces cellulases differ from the cellulases of *T. reesei* and *H. insolens* (except EGI/Cel7A), commonly used in denim finishing, as they do not contain a CBM. Cellulases from which the native CBM have been deleted, both bacterial (*Cellumonas fimi)* and fungal (*H. insolens)*, generally decrease indigo staining levels and cause less backstaining than do the intact enzymes (Andreaus *et al.*, 2000). Fowler *et al.* (2001) used *T. reesei* EGI and EGII catalytic core and CBHI core with EGIII (no CBD) and reported decreased backstaining. Cavaco-Paulo *et al.* (1998b) suggested that the prevention of backstaining during stone-washing requires an enzyme with very little affinity for indigo dye and reduced binding of the cellulase protein to the cotton cellulose. Thus the absence of a CBM might play a role in the low backstaining properties of the *Ma* cellulases. Cellulases of *T. reesei* cause higher indigo staining than those of *H. insolens* (Cavaco-Paulo *et al.*, 1998b). Campos *et al.* (2000) showed that cellulases of *T. reesei* have more affinity for plain indigo dye than the cellulases of *H. insolens.* The affinity of *M. albomyces* cellulases for indigo dye remains to be clarified. Gusakov *et al.* (2000b) suggested that certain cellulases may have hydrophobic domains (clusters of closely located non-polar residues) on their surface, and that these

may bind indigo and thus act as emulsifiers helping the dye to float out of cellulose fibres into the bulk solution. This might be one explanation for the observed phenomenon of reduced backstaining levels obtained when *Ma* Cel45A was used together with *Ma* Cel7A or *Ma* Cel7B.

4.5 Future perspectives

Industrially useful *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles were constructed. Some recent results of the molecular mechanisms of cellulase gene regulation and protein secretion in *T. reesei* could possibly be utilized to increase the production levels of cellulases further. As an example the overexpression of positively acting regulatory factors might be a tool to circumvent the possible titration of transcriptional factors or regulatory proteins in enhancing production of *T. reesei* EGs and CBHs with the aid of the *cbh1* promoter, leading to still more cost-effective levels of cellulase production. Deletion of the negative regulatory factor ACEI and overexpression of the positive regulatory factor ACEII of cellulase promoters could represent one possible way to increase the production of cellulases in *T. reesei* (Aro, 2003). However, deletion of *ace1* or overproduction of *ace2* did not significantly improve cellulase production levels of *T. reesei* ALKO2221 in industrially feasible medium (M. Paloheimo and J. Vehmaanperä, Roal Oy, personal communication). The cloning of genes involved in the secretion processes in *Trichoderma* has been started, with the aim of improved secretion. Thereby, the overall production levels of cellulases may possibly also be increased.

Backstaining is not a desired effect in denim wash. *Ma* Cel7A and Cel7B were able to decrease the backstaining level in denim finishing when used along with *Ma* Cel45A. This phenomenon requires further clarification. Better understanding of the mechanism behind reduction or prevention of backstaining by certain cellulases or cellulase mixtures will enable the design of optimal cellulase compositions having the desired denim finishing effect with high abrasion and no backstaining.

Other potential uses of the new cellulase preparations also need to be elucidated. Strains producing enhanced *T. reesei* EGII or EGI and EGII or the *Ma* Cel45A

(EGV) or Cel7A (EGI) activity can possibly be used for more economical production of β-glucanase for degradation of β-glucan in feed in order to improve the quality of feed. The suitability of the cellulases in efficient conversion of biomass to sugars that can be fermented to ethanol should also be investigated. Cellobiohydrolases are the key components of the redesigned, highly synergistic cellulase mixtures required for such processes (Teeri, 1997). Therefore the newly described CBH preparations as well as possibly the novel cellulases of *M. albomyces* could be used in applications in which total hydrolysis of cellulose is needed. *M. albomyces* cellulases could also be exploited in detergent applications, if they are found to be compatible with chemical components present in detergents. In addition to detergents, deinking is also a potential application due to the broad pH range of the cellulases and their ability to function in the alkaline pH range.

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